

# CAVELIER

ABOGADOS

27 27

www.cavelier.com  
cavelier@cavelier.com

EDIFICIO S  
CARRERA 4 N  
BOGOTA 8, CC

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

Teléfono: (57-1) 347 3611  
3650  
860  
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No. 04-111108- -00004-0000

Fecha: 2008-05-19 16:50:33 Dep. 2020 NUECREACIONE  
Tra. 11 PATENTEIYII Eve: 379 FASENACIONALI  
Act. 444 RESPUESTAREQUE Folios: 7

Señora Jefe de la  
**DIVISION DE NUEVAS CREACIONES**  
Superintendencia de Industria y Comercio  
E. S. D.

Trámite : 011  
Evento : 379  
Actuación : 444

Referencia : Expediente No. 04.111.108  
Asunto : Solicitud de Patente para **NUEVA SAL DE PERINDORPIL Y COMPOSICIONES FARMACEUTICAS QUE LA CONTIENEN.**  
Solicitante : **LES LABORATOIRES SERVIER**  
Clase : A61K 031/404, C07D, C07D 209/042

1. Yo, Emilio FERRERO, obrando como apoderado del solicitante en el asunto antes identificado, me refiero al Oficio **No. 397** notificado por fijación en lista del **11 de Enero de 2008**. Mediante memorial radicado el **27 de Marzo de 2008** solicité un **plazo de treinta (30) días para dar respuesta a este oficio.**

2. Mediante el Oficio **No. 397** se puso en conocimiento del solicitante el informe del examen de fondo que contiene las observaciones hechas por su Despacho sobre la presente solicitud de patente. Atiendo a las observaciones de la siguiente manera:

### **3. De la solicitud de patente :**

#### **3.1 Descripción (artículo 28 D.486)**

Su Despacho enuncia lo siguiente:

***“esta Dependencia se permite señalar que no existe evidencia que permita identificar el verdadero salto cualitativo en la regla técnica, dado que la***

***divulgación realizada del problema técnico y su solución es un mero acercamiento al estudio definitivo”***

Con respecto a lo señalado por su Despacho mi representada desea señalar lo siguiente:

Respetuosamente nos permitimos disentir de la observación del Señor Examinador debido a que en la especificación de la presente solicitud de patente se muestran datos comparativos entre la sal de arginina de perindopril y la sal de tert-butilamina de perindopril. Por tanto, tal como se encuentran divulgados en la especificación, los datos comparativos muestran claramente la gran estabilidad de la sal de arginina en comparación con la sal de tert-butilamina y por ende el salto técnico que representa la presente invención frente a lo conocido en el estado del arte.

Por tanto la objeción formulada a este respecto se debe tener por superada.

### **3.2 Reivindicaciones (artículo 30 D 486)**

De acuerdo al artículo 34 de la Decisión 486, se presenta un nuevo capítulo reivindicatorio como sustituto del anteriormente presentado.

En este nuevo capítulo reivindicatorio se elimina la reivindicación 5. De tal manera que el nuevo capítulo reivindicatorio esta constituido por cuatro (4) reivindicaciones.

De esta manera la siguiente objeción queda superada:

#### **3.2.1 Excepción a la patentabilidad ( artículo 20 D486 )**

La reivindicación 5 objetada fue eliminada del nuevo pliego reivindicatorio.

### **3.3 Evaluación de los requisitos de patentabilidad:**

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Su Despacho concluye que el nivel inventivo de las antiguas reivindicaciones 1 a 5 de la presente solicitud de patente, se ven afectadas por el documento WO 0187835 (D1)

Con respecto a que el documento D1 afecta el nivel inventivo de la presente solicitud, mi representada desea señalar lo siguiente:

El estado de la técnica más cercano (D1), describe una forma cristalina de la sal de tert-butilamina de perindopril que puede ser obtenida de una manera reproducible a escala industrial. Esta forma posee muy buenas características para la filtración, es fácil de secar y formular, es decir que se cumplió con el objetivo de esta invención.

Sin embargo, la estabilidad y las condiciones de almacenamiento de esta forma cristalina de perindopril son las mismas que eran conocidas en el estado del arte. Así entonces, si bien la forma cristalina revelada en D1 presenta ventajas a un nivel industrial, este documento no reclama ninguna ventaja en términos de estabilidad a diferencia de la sal de arginina de perindopril, reivindicada en la presente solicitud de patente.

Esta diferencia y la forma como se llegó a ella, sin embargo, no están ni enseñadas ni sugeridas en D1 y en consecuencia, para una persona versada en la materia correspondiente, esta invención no podía resultar obvia o derivarse de manera evidente del documento referenciado por su Despacho.

Por lo tanto, el documento D1 no puede tenerse como una anterioridad válida en materia de nivel inventivo y por ende guarda silencio con respecto a las ventajas en términos de estabilidad de la sal de arginina de perindopril y sus hidratos así como sus composiciones farmacéuticas.

Puesto que se han atendido a cabalidad las observaciones hechas por su Despacho sobre la presente solicitud de patente, respetuosamente solicito se continúe con su tramitación.

**FORMULARIO UNICO DE CORRECCIONES Y MODIFICACIONES**

|                                                  |                                                                                                                                                                                                                                                                                                                                                                                            |                                                                                                                                                                                            |
|--------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>1</b>                                         | <b>SOLICITUD DE:</b><br><input type="checkbox"/> Corrección de Error Material <input checked="" type="checkbox"/> Modificación a una solicitud                                                                                                                                                                                                                                             |                                                                                                                                                                                            |
| <b>2</b><br><b>SOLICITANTE</b>                   | <b>Nombre:</b> LES LABORATORIES SERVIER<br>Sociedad constituida y existente bajo las leyes de <b>Francia.</b><br><b>Dirección:</b> 12, Place de la Défense,<br>92415 Courbevoie Cedex,<br>Francia<br><b>Domicilio:</b> Courbevoie Cedex,<br>Francia<br><b>Teléfono:</b> 546 09 70 <b>Fax:</b> 249 40 70<br><b>E-mail:</b> <a href="mailto:clientes@cavelier.com">clientes@cavelier.com</a> | <b>IDENTIFICACION</b><br>C.C. <input type="checkbox"/> NIT <input type="checkbox"/><br>C.E. <input type="checkbox"/> Otro <input type="checkbox"/><br>Número: _____                        |
| <b>3</b><br><b>REPRESENTANTE<br/>O APODERADO</b> | <b>Nombre:</b> Emilio FERRERO<br><b>Dirección:</b> Carrera 4ª No. 72-35<br><b>Teléfono:</b> 347 36 11 <b>Fax:</b> 217 92 11<br><b>E-mail:</b> <a href="mailto:cavelier@cavelier.com">cavelier@cavelier.com</a>                                                                                                                                                                             | <b>IDENTIFICACION</b><br>C.C. <input checked="" type="checkbox"/> NIT <input type="checkbox"/><br>C.E. <input type="checkbox"/> Otro <input type="checkbox"/><br>Número: 438.019 TP 31.929 |
| <b>4</b>                                         | <b>Número de expediente:</b> <u>04.111.108</u>                                                                                                                                                                                                                                                                                                                                             |                                                                                                                                                                                            |
| <b>5</b>                                         | <b>Descripción de la corrección o modificación solicitada:</b><br><u>Presento nuevo capítulo reivindicatorio compuesto por 4 reivindicaciones</u><br><u>para superar las objeciones y solicito que el examen de patentabilidad se</u><br><u>realice sobre estas nuevas reivindicaciones presentadas.</u>                                                                                   |                                                                                                                                                                                            |
| <b>6</b>                                         | <b>Comprobante de pago No.</b> <u>08-35,705</u> <b>Fecha:</b> <u>6 de mayo de 2008</u>                                                                                                                                                                                                                                                                                                     |                                                                                                                                                                                            |
| <b>7</b>                                         | <b>Anexos</b><br><input checked="" type="checkbox"/> Comprobante de pago de la tasa por \$103.000.00 por concepto de modificaciones.<br><input type="checkbox"/> Poderes, si fuere el caso<br><input type="checkbox"/> Documento que acredita la existencia y representación legal de las partes, cuando sean personas jurídicas.                                                          |                                                                                                                                                                                            |
| <b>8</b>                                         | <b>NOMBRE:</b> EMILIO FERRERO                                                                                                                                                                                                                                                                                                                                                              | <b>FIRMA:</b>                                                                                          |
| C.C. 438.019 de Usaquén T.P.A. 31.929            |                                                                                                                                                                                                                                                                                                                                                                                            |                                                                                                                                                                                            |

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### REIVINDICACIONES.

1. La sal de arginina de perindopril y sus hidratos.
2. La composición farmacéutica que comprende, o ingrediente activo, la sal de arginina de perindopril y sus hidratos, en combinación con uno o más excipientes farmacéuticamente aceptables.
3. Composición farmacéutica de acuerdo con la reivindicación 2, caracterizada por que esta se presenta en forma de una tableta de liberación inmediata.
4. Composición farmacéutica de acuerdo a la reivindicación 2 o reivindicación 3, caracterizada por que esta contiene de 0,2 a 10 mg de sal de arginina de perindopril.

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 800176089-2

RECIBO OFICIAL DE CAJA : 08 - 35,705  
FECHA : MAYO 6 DE 2008

\*\*\*\*\* CONSIGNACION \*\*\*\*\*

| DEPOSITANTE       | TIPO PAGO    | BANCO           | CUENTA    | No. PAGO | FECHA PGO  | Vr. PAGO      |
|-------------------|--------------|-----------------|-----------|----------|------------|---------------|
| CAVELIER ABOGADOS | CONSIGNACION | BANCO DE BOGOTA | 062754387 | 773183   | 06/05/2008 | 40,376,500.00 |

\*\*\*\*\* CONCEPTO \*\*\*\*\*

| CANT. | RENTISTICO  | CONCEPTO                       | TOTAL CONCEPTO |
|-------|-------------|--------------------------------|----------------|
| 1     | 50005-01-03 | CAMBIO DE MODALIDAD Y MODIFICA |                |
|       |             | 12 MARCAS Y LEMAS COMERCIALES  | 103,000.00     |
|       |             | TOTAL :                        | \$ 103,000.00  |

SON: CIENTO TRES MIL PESOS

RESPONSABLE : \_\_\_\_\_

Colo 14526-841-001-9

RECIBO DE CAJA APLICADO AL EXPEDIENTE No. \_\_\_\_\_

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Europäisches Patentamt  
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**/(A61K45/06,31:505),**  
**(A61K37/64,31:505)**

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(71) Applicant: **JANSSEN PHARMACEUTICA N.V.**  
**Turnhoutseweg 30**  
**B-2340 Beerse(BE)**

(72) Inventor: **Assogna, Giuseppe**  
**Via Cechov 107**  
**Rome(IT)**  
Inventor: **Lucchetti, Giovanni**  
**Via Mengoni 7**  
**Rome(IT)**

(84) **Synergistic compositions containing ketanserin.**

(87) **Pharmaceutical compositions comprising ketanserin and an ACE inhibitor in a quantity producing a synergistic antihypertensive effect. A process of preparing such synergistic compositions.**

**EP 0 391 462 A1**

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## SYNERGISTIC COMPOSITIONS CONTAINING KETANSERIN

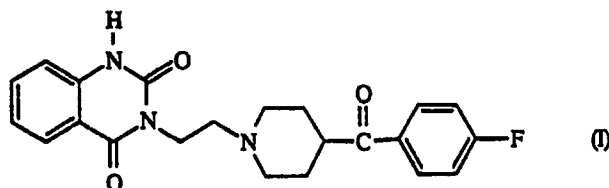
Background of the invention.

Several classes of chemical compounds are known as antihypertensives. Among these classes a group of piperidinylalkyl quinazolinones having antiserotonergic activity is of particular interest. These compounds inhibit serotonin-induced vasoconstriction and are useful as antihypertensives. Another class of known antihypertensives are the angiotensin converting enzyme (ACE) inhibitors, which exert their influence by inhibiting ACE, the enzyme mediating the conversion of angiotensin I to angiotensin II, a vasoconstrictive hormone, and the degradation of the vasodilator kinin peptide (bradykinin) to its inactive metabolites. Further, there are known antihypertensive compositions comprising ACE inhibitors and other antihypertensive agents, said compositions showing an additive effect on the lowering of the blood pressure. For example, in U.S. Pat. No. 4,217,347 there are described compositions comprising ACE inhibitors and diuretics and in EP-0,288,732, compositions of ACE inhibitors with calcium channel blockers. The antihypertensive effect of ketanserin and captopril was reported on the International Symposium on Serotonin : From cell biology to pharmacology and therapeutics, Florence, Italy, March 29 - April 1, 1989. It has now been found that the compound ketanserin in combination with ACE inhibitors synergistically acts on the lowering of the blood pressure.

Description of the invention.

The present invention is concerned with synergistic compositions comprising a pharmaceutically acceptable carrier and as active ingredients an antihypertensively effective amount of the compound ketanserin or a pharmaceutically acceptable acid addition salt form thereof and an ACE inhibitor or a pharmaceutically acceptable acid or base addition salt form thereof.

Ketanserin as referred to hereinabove is the generic name of the compound 3-[2-[4-(4-fluorobenzoyl)-1-piperidinyl]ethyl]-2,4(1H,3H)-quinazolinone, which may be represented by the formula



This compound, its synthesis as well as its antiserotonergic properties are described in U.S. Pat. No. 4,335,127, incorporated herein by reference.

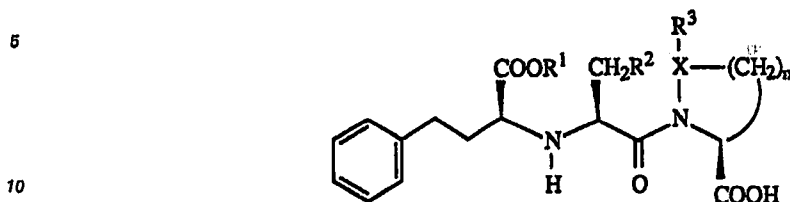
The term ACE inhibitor as used hereinabove defines chemical compounds which inhibit angiotensin converting enzyme (ACE), also known as kinase II. Typical examples of ACE inhibitors are the compounds mentioned in U.S. Pat. Nos 4,399,136, 4,487,929, 4,374,131, 4,416,831, 4,473,575, Eur. Pat. No. 0,072,352; in particular benazepril; U.S. Pat. No. 4,046,889, in particular captopril; Eur. Pat. No. 0,012,401, in particular enalapril, enalaprilat and lisinopril; U.S. Pat. No. 4,248,883, in particular alacepril; U.S. Pat. Nos 4,385,051 and 4,474,692, in particular delapril, Great Britain Pat. No. 2,102,412, in particular moveltipril; U.S. Pat. No. 4,727,160, in particular ramipril and ramiprilat; U.S. Pat. No. 4,512,924, in particular cilazapril and cilazaprilat; U.S. Pat. No. 4,344,949, in particular quinapril and quinaprilat; Eur. Pat. No. 0,083,896, in particular fosinopril; Eur. J. Clin. Pharmacol., 1987, 31, 519, in particular perindopril and perindoprilat; and also the compounds indolapril, spirapril, spiraprilat and zofenopril. These groups of active ingredients are listed with the purpose of providing representative examples and are not intended to restrict the scope of the present invention.

Particular compositions are those synergistic compositions wherein the ACE inhibitor is one of the agents pertaining to the patents or publication cited hereinabove, and more in particular the agents specifically mentioned hereinabove.

A first group of more particular compositions comprises those particular compositions wherein the ACE inhibitor is selected from captopril, lisinopril, enalapril and cilazapril.

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Another group of more particular compositions comprises those particular compositions wherein the ACE inhibitor is a compound that contains the moiety



- wherein R¹ is hydrogen or ethyl;  
R² is hydrogen or aminopropyl;  
15 R³ is hydrogen; or R² and R³ taken together form 1,2-ethanediyl;  
X is CH or N; and  
n is 2 or 3.

The active ingredient ketanserin (I) may be present in the free base form or in a pharmaceutically acceptable acid addition salt form, the latter being obtained by reaction of the base form with an appropriate acid. Appropriate acids comprise, for example, inorganic acids such as hydrohalic acids, e.g. hydrochloric or hydrobromic acid; sulfuric acid; nitric acid; phosphoric acid and the like; or organic acids such as, for example, acetic, propanoic, hydroxyacetic, 2-hydroxypropanoic, 2-oxopropanoic, ethanedioic, propanedioic, butanedioic, (Z)-butenedioic, (E)-butenedioic, 2-hydroxybutanedioic, 2,3-dihydroxybutanedioic, 2-hydroxy-1,2,3-propanetricarboxylic, methanesulfonic, ethanesulfonic, benzenesulfonic, 4-methylbenzenesulfonic, cyclohexanesulfamic, 2-hydroxybenzoic, 4-amino-2-hydroxybenzoic and the like acids. A particularly interesting acid addition salt form of the compound ketanserin (I) is (+)-3-[2-[4-(4-fluorobenzoyl)-1-piperidiny]-ethyl]-2,4(1H,3H)-quinazolinone R-(R\*,R\*)-2,3-dihydroxybutanedioate (1:1).

The ACE inhibitor may be present in its neutral or zwitterionic form or in a pharmaceutically acceptable acid or base addition salt form, the latter being obtained by reaction of the neutral or zwitterionic form with respectively an appropriate acid as defined hereinabove, or with an appropriate base. Appropriate base addition salt forms comprise, for example, the ammonium salts, the alkali and earth alkaline metal salts, e.g., the sodium, potassium, magnesium, calcium salts and the like, salts with organic bases, e.g., the benzathine, N-methyl-D-glucamine, hydrabamine salts, and salts with amino acids such as, for example, arginine, lysine and the like.

35 A particular salt form may be obtained by reacting the base form of the compound ketanserin (I) with the acid form of an ACE inhibitor as defined hereinabove.

The term addition salt as used hereinabove also comprises the solvates which the compound ketanserin, the ACE inhibitors defined hereinabove as well as the salts of ketanserin with said ACE inhibitors, are able to form. Said solvates are meant to be included within the scope of the present invention. Examples of such solvates are e.g., the hydrates, alcoholates and the like.

40 The compositions comprising the compound ketanserin (I) and an ACE inhibitor unexpectedly show a synergistic effect on the lowering of the blood pressure. The decrease in systolic and diastolic blood pressure (SBP ; DBP) observed in combination therapy with both ketanserin (I) and an ACE inhibitor is larger than the sum of the decreases in SBP and DBP observed in monotherapy with either ketanserin or an ACE inhibitor when applied at the same rate. As a consequence a combination therapy using compositions with lower doses of each drug than used normally in monotherapy, may be effective, and occasional side-effects associated with the larger amounts used in monotherapy may be reduced or prevented altogether. Additionally, a combination therapy using a dose of each drug as used normally in monotherapy, has the advantage that an effective lowering of the blood pressure may be obtained in the often large number of patients who do not respond to conventional monotherapy.

50 A further advantage of the present compositions appears to be the fact that therapy with most ACE inhibitors is accompanied with mild potassium retention (hyperkalaemia). It is known that ketanserin therapy may prove unfavorable and should be avoided in patients with a low potassium blood level. This property of ketanserin limits its utility in the treatment of hypokalaemic patients and also limits possible combination therapies of ketanserin with other drugs which may provoke hypokalaemia such as, for example, antihypertensives, in particular diuretics. The mild hyperkalaemic effect of ACE inhibitors obviously enhances the safety and utility of ketanserin therapy, particularly in patients prone to hypokalaemia.

55 The amount of each of the active ingredients ketanserin (I) in the free base form and an ACE inhibitor in

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the neutral or zwitterionic form in the synergistic compositions according to the present invention is such that a synergistic antihypertensive effect is obtained upon administration. For example, the amount of the active ingredient ketanserin (I) in said synergistic compositions may typically range from about 5 to about 150 mg, particularly from about 10 to about 80 mg and more particularly from about 20 to about 40 mg per dosage unit form. ACE inhibitors, such as those pertaining to the patents or publication cited hereinabove and particularly the ACE inhibitors specifically mentioned hereinabove, differ both in molecular weight and in activity and therefore may be administered in fairly different quantities. As a consequence, the amount of ACE inhibitor in said synergistic compositions depends on the nature of said ACE inhibitor and in general ranges from about 1 to about 300 mg, particularly from about 5 to about 150 mg and more particularly from about 10 to about 50 mg per dosage unit form.

In said synergistic compositions the molar ratio between the active ingredient ketanserin (I) in the free base form and the ACE inhibitor in the neutral or zwitterionic form may vary depending both on the nature of the ACE inhibitor and the application aimed at. The molar ratio of the active ingredient ketanserin (I) to the ACE inhibitor will be such that a synergistic antihypertensive effect is obtained and in particular may be situated between 50:1 and 1:50, more in particular between 20:1 to 1:20 or between 10:1 and 1:10, most particularly between 5:1 and 1:5. In the synergistic compositions according to the present invention wherein an ACE inhibitor with low molecular weight and moderate activity, such as, for example, captopril is used, the molar ratio of the active ingredient ketanserin (I) to said ACE inhibitor may be situated between 5:1 and 1:30, in particular between 1:1 to 1:5 and more particularly between 1:2 and 1:3, or between 1:1 and 1:1.5. If, on the contrary a potent ACE inhibitor with relatively high molecular weight, such as, for example, enalapril, lisinopril, cilazapril or ramipril is used in the synergistic compositions according to the present invention, the molar ratio of the active ingredient ketanserin (I) to said ACE inhibitor may be situated between 50:1 and 1:5, in particular between 10:1 to 1:1 and more particularly between 5:1 and 3:1.

To prepare the pharmaceutical compositions of this invention, an effective amount of the active ingredients, in acid or base addition salt form or base form, is combined in intimate admixture with a pharmaceutically acceptable carrier, which can take a wide variety of forms depending on the form of preparation desired for administration. These pharmaceutical compositions are desirably in unitary dosage form suitable, preferably, for administration orally, rectally, percutaneously, or by parenteral injection. For example, in preparing the compositions in oral dosage form, any of the usual pharmaceutical media may be employed, such as, for example, water, glycols, oils, alcohols and the like in the case of oral liquid preparations such as suspensions, syrups, elixirs and solutions; or solid carriers such as starches, sugars, kaolin, lubricants, binders, disintegrating agents and the like in the case of powders, pills, capsules and tablets. Because of their ease in administration, tablets and capsules represent the most advantageous oral dosage unit form, in which case solid pharmaceutical carriers are obviously employed. For parenteral compositions, the carrier will usually comprise sterile water, at least in large part, though other ingredients, for example, to aid solubility, may be included. Injectable solutions, for example, may be prepared in which the carrier comprises saline solution, glucose solution or a mixture of saline and glucose solution. Injectable suspensions may also be prepared in which case appropriate liquid carriers, suspending agents and the like may be employed. In the compositions suitable for percutaneous administration, the carrier optionally comprises a penetration enhancing agent and/or a suitable wettable agent, optionally combined with suitable additives of any nature in minor proportions, which additives do not cause any significant deleterious effects on the skin. Said additives may facilitate the administration to the skin and/or may be helpful for preparing the desired compositions. These compositions may be administered in various ways, e.g., as a transdermal patch, as a spot-on or as an ointment. Acid addition salts of (I) due to their increased water solubility over the corresponding base form, are obviously more suitable in the preparation of aqueous compositions.

It is especially advantageous to formulate the aforementioned pharmaceutical compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used in the specification and claims herein refers to physically discrete units suitable as unitary dosages, each unit containing a predetermined quantity of active ingredient calculated to produce the desired therapeutic effect, in association with the required pharmaceutical carrier. Examples of such dosage unit forms are tablets (including scored or coated tablets), capsules, pills, powder packets, wafers, injectable solutions or suspensions, teaspoonfuls, tablespoonfuls and the like, and segregated multiples thereof.

The present invention also concerns a method of lowering the blood pressure in warm-blooded animals suffering from hypertension or in need of blood pressure reducing medication, said method comprising administering to said warm-blooded animals an antihypertensively effective amount of the compound ketanserin (I) and an ACE inhibitor as defined hereinabove. The amount of each of the active ingredients ketanserin (I) and an ACE inhibitor in the method according to the present invention is such that a synergistic antihypertensive effect is obtained upon administration to said warm-blooded animals.

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The compound ketanserin (I) or a pharmaceutically acceptable acid addition salt thereof may be administered before, during or after the administration of the ACE inhibitor, provided that the time between the administration of the compound ketanserin (I) and the administration of the ACE inhibitor is such that ketanserin (I) and the ACE inhibitor are allowed to act synergistically on the lowering of the blood pressure.

5 When simultaneous administration of the compound ketanserin (I) and an ACE inhibitor is envisaged, a composition containing both an ACE inhibitor and the compound ketanserin (I) may be particularly convenient. Or, the compound ketanserin (I) and the ACE inhibitor may be administered separately in the form of suitable compositions. The latter can be prepared as described hereinabove.

The present invention also comprises products containing the compound ketanserin (I) and an ACE inhibitor as a combined preparation for simultaneous, separate or sequential use in antihypertension therapy. Such products may comprise, for example, a kit comprising a container with a suitable composition containing ketanserin (I) or a pharmaceutically acceptable acid addition salt thereof and another container containing a composition with an ACE inhibitor. Such a product may have the advantage that a physician wishing to administer antihypertension therapy, can select on the basis of the diagnosis of the patient to be

15 treated, the appropriate amounts of each component and the sequence and timing of the administration thereof.

Those of skill in treating warm-blooded animals suffering from hypertension or warm-blooded animals in need of blood-pressure reducing medication, could easily determine the effective amounts of both active ingredients from the results presented hereinafter. In general it is contemplated that an effective daily dose of the compound ketanserin (I) or its pharmaceutically acceptable acid addition salts would be from about 0.04 mg to about 4 mg/kg bodyweight, in particular from about 0.1 mg to about 2 mg/kg bodyweight and preferably from about 0.2 to about 1 mg/kg bodyweight, in combination with an effective daily dose of an ACE inhibitor from about 0.01 mg to about 10 mg/kg bodyweight, in particular from about 0.1 mg to about 5 mg bodyweight and preferably from about 0.2 to about 2 mg/kg bodyweight. It may be appropriate to

25 administer the required dose as two, three, four or more sub-doses at appropriate intervals throughout the day. Said sub-doses may be formulated as unit dosage forms.

The following examples are intended to illustrate and not to limit the scope of the present invention in all its aspects.

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#### A. Clinical Examples.

##### Example 1

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The effect of a combination of the compound ketanserin (I) and an ACE inhibitor, captopril, on the lowering of blood pressure was investigated in a double-blind randomized cross-over study according to a latin square design.

Adult ( $\geq 18$  years) patients with moderate, uncomplicated hypertension ( $105 \text{ mmHg} \leq \text{DBP} \leq 115 \text{ mmHg}$  at the end of a 1 month placebo wash-out period) were randomly assigned to: - placebo (1 tab q.i.d.);

40 - placebo (1 tab b.i.d.) + ketanserin (1 tab 40 mg b.i.d.);

- placebo (1 tab b.i.d.) + captopril (1 tab 50 mg b.i.d.);

- ketanserin (1 tab 40 mg b.i.d.) + captopril (1 tab 50 mg b.i.d.).

At the end of each treatment period (1-2 hours after the last dose), blood pressure (by a mercury sphygmomanometer) and heart rate (by the palpatory method over a 30" period) of patients seated for 10' were recorded by the same investigator during 3 measurements at 2' intervals.

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The following table shows the difference of systolic and diastolic blood pressure (SBP, DBP) and the heart rate (HR) (mean values  $\pm$  standard error) in mmHg, respectively b.p.m., between treated and untreated patients.

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|     | Baseline    | Placebo    | Placebo + Ketanserin | Placebo + Captopril | Ketanserin + Captopril |
|-----|-------------|------------|----------------------|---------------------|------------------------|
| SBP | 167.92±3.51 | +3.75±4.27 | -8.33±2.33           | -8.75±1.96          | -22.08±3.61            |
| DBP | 107.5±0.97  | -1.07±1.7  | -7.92±1.3            | -8.75±1.3           | -17.08±1.89            |
| HR  | 78.17±3.31  | -2.75±3.07 | -1.92±2.2            | -3.33±2.85          | -4.83±2.65             |

Example 2

The effect of a combination of the compound ketanserin (I) and enalapril on the lowering of blood pressure was investigated in a randomized double-blind parallel group study. Elderly ( $\geq 50$  years) patients with mild to moderate, uncomplicated, essential hypertension ( $95 \text{ mm Hg} \leq \text{DBP} \leq 105 \text{ mm Hg}$  at the end of a 3 month period on double blind monotherapy) received ketanserin (1 tab 40 mg b.i.d.) + enalapril (1 tab 10 mg b.i.d.). The results of the preliminary 3 month monotherapy phase (Ketanserin : 2 weeks, 1 tab 20 mg b.i.d., 10 weeks, 1 tab 40 mg b.i.d. or Enalapril: 12 weeks, 1 tab 10 mg b.i.d.) and the combination phase (4 weeks) are shown in the table below (16 patients). Bloodpressure (SBP, DBP) and heart rate (HR) (mean values  $\pm$  standard error) are in mm Hg and b.p.m.

|     | End Placebo<br>runin | End Monotherapy Phase |                   | End Combination Phase  |             |
|-----|----------------------|-----------------------|-------------------|------------------------|-------------|
|     |                      | Ketanserin (9<br>pts) | Enalapril (7 pts) | Ketanserin + Enalapril |             |
|     |                      |                       |                   | (9 pts)                | (7 pts)     |
| SBP | 163.12±11.52         | 148.88±13.64          | 160.0±5.77        | 144.44±9.82            | 152.85±4.87 |
| DBP | 109.06±5.23          | 102.22±5.65           | 101.42±10.07      | 89.44±7.26             | 90.71±3.45  |
| HR  | 74.68±5.37           | 74.44±4.36            | 75.42±6.21        | 74.66±4.27             | 74.42±5.53  |

B. Composition Examples.

Typical pharmacological formulations in dosage unit form suitable for administration to animal and human subjects in accordance with the present invention, are those formulations described in U.S. Pat. No. 4,335,127 for the compound ketanserin (I) and those formulations wherein the active ingredient is an ACE inhibitor pertaining to one of the patents cited hereinabove, more particularly those wherein the active ingredient is one of the agent specifically mentioned hereinabove, all said patents being incorporated herein by reference for the description of said formulations. Especially convenient formulations are those formulations of the active ingredients which are commercially available.

The following formulations exemplify typical new pharmaceutical compositions in dosage unit form comprising both the compound ketanserin (I) and captopril, suitable for administration to animal and human subjects in accordance with the present invention.

Example 2 : Capsules

The following formulation provides 1000 capsules each comprising as the active ingredient 10 mg of the compound ketanserin (I) and 10 mg of captopril as defined hereinabove:

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|                             |           |
|-----------------------------|-----------|
| ketanserin :                | 10 grams  |
| captopril :                 | 10 grams  |
| sodium lauryl sulfate :     | 6 grams   |
| corn starch :               | 56 grams  |
| lactose :                   | 56 grams  |
| colloidal silicon dioxide : | 0.8 grams |
| magnesium stearate :        | 1.2 grams |

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All ingredients are mixed together well. The resulting mixture is filled into suitable hardened gelatine capsules.

15 Example 3 : Film-coated tablets

10,000 compressed tablets, each containing as the active ingredients 20 mg of the compound ketanserin (I) and 25 mg of captopril, may be prepared from the following formulation:

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|                                         |           |
|-----------------------------------------|-----------|
| ketanserin :                            | 200 grams |
| captopril :                             | 250 grams |
| starch :                                | 400 grams |
| lactose :                               | 890 grams |
| polyvinylpyrrolidone (Kollidon-K 90®) : | 20 grams  |
| microcrystalline cellulose (Avicel®) :  | 200 grams |
| sodium dodecyl sulfate :                | 10 grams  |
| hydrogenated vegetable oil :            | 30 grams  |

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A mixture of the active ingredients ketanserin (I) and captopril, the lactose and the starch is mixed by vigorous stirring and is thereafter humidified with a solution of sodium dodecyl sulfate and the polyvinylpyrrolidone in about 200 ml of purified water. The wet powder is sieved, dried and sieved again. Then there is added the microcrystalline cellulose and the hydrogenated vegetable oil. The whole is mixed well and compressed into tablets. The tablets are coated with a solution methyl cellulose (Methocel) including a colour, following art-known coating procedures.

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40 Example 4 : Injectable solution

1 liter of a parenteral solution comprising 5 mg/ml of ketanserin and 10 mg/ml of captopril may be obtained from:

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|                                               |            |
|-----------------------------------------------|------------|
| ketanserin :                                  | 5 grams    |
| captopril :                                   | 10 grams   |
| propylene glycol :                            | 0.05 grams |
| methyl 4-hydroxybenzoate :                    | 1.8 grams  |
| propyl 4-hydroxybenzoate :                    | 0.2 grams  |
| purified water for injection q.s. ad 1 liter. |            |

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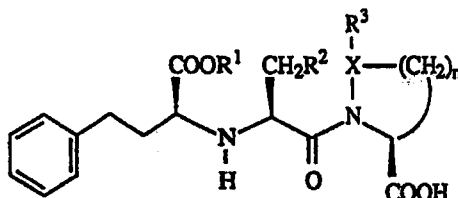
The methyl and propyl 4-hydroxybenzoates are dissolved in about 0.5 l of boiling water for injection. The solution is cooled to about 50°C and the active ingredients and the propylene glycol are added while stirring. The solution is allowed to cool to room temperature and is supplemented with water for injection q.s. ad 1 liter. The solution is sterilized by filtration (U.S.P. XVII, p 811) and filled in sterile containers.

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# Claims

1. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and as active ingredients the compound ketanserin or a pharmaceutically acceptable acid addition salt form thereof and an ACE inhibitor or a pharmaceutically acceptable acid or base addition salt form thereof in a quantity producing an antihypertensive synergistic effect.
2. A composition according to claim 1 wherein the ACE inhibitor is selected from benazepril, captopril, enalapril, enalaprilat, lisinopril, alacepril, delapril, moxetipril, ramipril, ramiprilat, perindopril, perindoprilat, quinapril, quinaprilat, cilazapril, cilazaprilat, fosinopril, indolapril, spirapril, spiraprilat and zofenopril.
3. A composition according to claim 1 wherein the ACE inhibitor is a compound of formula



- wherein R¹ is hydrogen or ethyl;  
R² is hydrogen or aminopropyl;  
R³ is hydrogen; or R² and R³ taken together form 1,2-ethanediyl;  
X is CH or N; and  
n is 2 or 3.
4. A composition according to any of claims 1 to 3 wherein the molar ratio of the compound ketanserin to the ACE inhibitor ranges from about 10:1 to about 1:10.
5. A composition according to claim 4 wherein the molar ratio ranges from about 5:1 to about 1:5.
6. A composition according to claim 1 wherein the molar ratio of ketanserin to captopril ranges from 1:1 to 1:1.5.
7. A composition according to claim 1 wherein the molar ratio of ketanserin to enalapril or to cilazapril ranges from 10:1 to 1:1.
8. A process for preparing a synergistic composition as claimed in any of claims 1 to 7, characterized in that the active ingredients are intimately mixed with a carrier.
9. A product containing the compound ketanserin or a pharmaceutically acceptable acid addition salt form thereof and an ACE inhibitor or pharmaceutically acceptable acid or base addition salt form thereof, as a combined preparation for simultaneous, separate or sequential use in antihypertension therapy.
10. A product according to claim 9 wherein the ACE inhibitor is as defined in claims 2 or 3.



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(71) Applicant: **SANDOZ LTD.**  
**Lichtstrasse 35**  
**CH-4002 Basel (CH)**

(72) Inventor: **Ross, Bruce Allan**  
**401 Ridge Road**  
**Fredon, N.J. 07860 (US)**  
Inventor: **Vivilecchia, Richard Victor**  
**155 Andrea Drive**  
**Rockaway, N.J. 07866 (US)**

(54) **Stabilized pharmaceutical compositions.**

(57) The invention relates to the use of certain acid donors as stabilizers in pharmaceutical compositions, and to the pharmaceutical compositions resulting therefrom.

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The present invention relates to the use of certain acid donors as stabilizers in pharmaceutical compositions, and to the pharmaceutical compositions resulting therefrom.

### Background of the Invention

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There are a number of pharmaceutical compositions which suffer from instability problems due to the fact that the active component is susceptible to certain types of degradation, thereby diminishing their attractiveness and, in some cases, rendering them unsuitable from a commercial standpoint. For example, several ACE (Angiotensin Converting Enzyme) inhibitor-containing compositions suffer from this drawback since certain ACE inhibitors degrade readily in pharmaceutical dosage forms. More particularly, and as is the case with other ACE inhibitors such as Quinapril and Enalapril, Spirapril may degrade in dosage forms to the diketo piperazine (the internal cyclization product) and the diacid (the ester hydrolysis product). Accordingly, in view of their usefulness in treating hypertension, a number of research endeavors have been directed to overcoming the instability problem associated with ACE inhibitor-containing compositions.

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### Description of the prior art

European Patent Application 264,888 is directed to the stabilization of ACE inhibitor-containing pharmaceutical compositions employing ascorbic acid alone or a combination of ascorbic acid with fumaric acid, maleic acid and/or citric acid as the stabilizing component(s).

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USP 4,743,450 is also directed to the stabilization of ACE inhibitor-containing pharmaceutical compositions employing, as the stabilizer component, a combination of an alkali or alkaline earth metal salt (preferably, magnesium carbonate) and a saccharide (preferably, mannitol or lactose).

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Although each of the above patents represents an attempt to overcome the instability problems associated with ACE inhibitor-containing compositions, there still exists a dire need for ACE inhibitor-containing compositions exhibiting improved stability, especially in the presence of moisture. To this end, the present invention is directed to pharmaceutical compositions, particularly ACE inhibitor-containing compositions, exhibiting improved stability.

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### DESCRIPTION OF THE INVENTION

Accordingly in one aspect the present invention provides

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a) a pharmaceutical composition comprising an active component liable to degradation and a hydrochloric acid donor, and

b) a process for the production of a stabilized pharmaceutical composition which comprises working up an active component liable to degradation with a hydrochloric acid donor, and

c) use of a hydrochloric acid donor to stabilize an active component liable to degradation and/or use of a hydrochloric acid donor as stabilizing agent in the manufacture of a pharmaceutical composition containing an active component liable to degradation, and

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d) a method of stabilizing a pharmaceutical composition as defined above containing an active component liable to degradation comprising incorporating therein a stabilizing effective amount of a hydrochloric acid donor.

The pharmaceutical compositions of the instant invention may exhibit advantages as follows:-

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1) The active component, e.g. an ACE inhibitor, may be preserved from degradation, and/or

2) They may exhibit an extended shelf-life under normal storage conditions, and/or

3) They may be less sensitive to moisture and even the stability may improve with an increase in moisture, and/or

4) They may exhibit less discoloration over a significant period of time, and/or

5) They may exhibit less instability when employed in the presence of colorants.

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In comparison to certain acidifiers which have previously been employed as stabilizers in pharmaceutical compositions, e.g. citric acid, maleic acid, ascorbic acid, etc., preferably the acid donors are chosen which release the more volatile hydrochloric acid and, therefore, effect a greater diffusion through the dosage form matrix. Although any compounds which produce hydrochloric acid would be suitable in the practice of the instant invention, preferred acid donors include an amino acid hydrochloride, such as glycine hydrochloride, glutamic acid hydrochloride, betaine hydrochloride, alanine hydrochloride, valine hydrochloride, lysine hydrochloride, arginine hydrochloride, aspartic acid hydrochloride and a Lewis acid chloride, such as ferric chloride, zinc chloride and aluminium chloride.

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The more preferred acid donors are glycine hydrochloride, glutamic acid hydrochloride and betaine hydrochloride.

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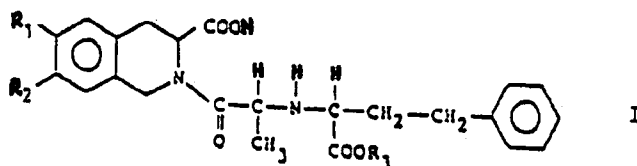
rochloride. The most preferred acid donor is glycine hydrochloride.

Other preferred hydrochloric acid donors may be chosen e.g. on the basis of vapour pressure measurements determining the release of hydrochloric acid, e.g. having similar properties to the preferred acid donors mentioned above.

6 Although, in general, the hydrochloric acid donor may be employed in any amount which will prevent degradation of the active component, e.g. as indicated by standard stability tests, e.g. an ACE inhibitor, the amount of the hydrochloric acid donor employed is between 1% and 25%, preferably between 1% and 20%, more preferably between 1% and 15%, e.g. from 1 to 10%, or 1 to 5%, e.g. 2% based on the total weight of the pharmaceutical composition.

10 The weight ratio of active component to the hydrochloric acid donor may be determined in conventional manner. The preferred weight ratio of active component to the hydrochloric acid donor is 2.5:1 to 1:7, more preferably 2:1 to 1:2.

The instant invention, viz., the use of a select group of hydrochloric acid donors as stabilizers in pharmaceutical compositions, applies to all active components in pharmaceutical compositions where buffering to a low pH for required stability is desired. It may be of particular interest for active components which are in acid addition salt form, e.g. hydrochloride salt form. The invention has particularly been found useful when applied to ACE inhibitor-containing pharmaceutical compositions since, as indicated above, many ACE inhibitors degrade readily in pharmaceutical dosage forms. In general, ACE inhibitor-containing pharmaceutical compositions wherein ACE inhibitor employed is prone to form diketopiperazine degradation products would benefit from the use of a select group of hydrochloric acid donors as stabilizers therefor. For example, one class of ACE inhibitors to which the instant invention would apply are compounds of formula I:

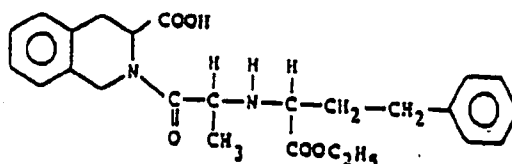


30 wherein

$R_1$  and  $R_2$ , independently, are hydrogen or a group  $-OC_nH_{2n+1}$ , where  $n$  is 1 to 5; and

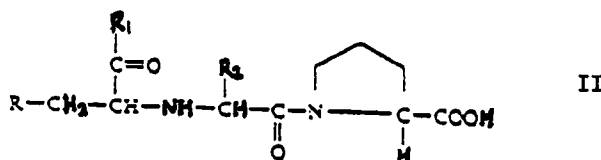
$R_3$  is hydrogen or a group  $-C_nH_{2n+1}$ , where  $n$  is as defined above.

In the above formula, preferred compounds are those where  $R_1$  and  $R_2$  have the same significance. More preferred compounds of the above formula are those where  $R_1$  and  $R_2$  are both hydrogen or methoxy and  $R_3$  is hydrogen or methyl. The most preferred compound of the above formula is Quinapril having the formula



45 All of the above compounds are known, having been previously described e.g. in USP 4,344,949. Moreover, their usefulness in treating hypertension as well as methods for their preparation are set forth therein.

Another class of ACE inhibitors to which the invention would apply are compounds of formula II:



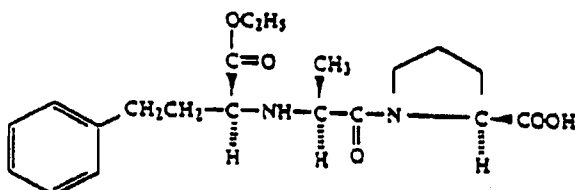
wherein

R is C<sub>1</sub>-C<sub>6</sub>alkyl, benzyl, benzylthio, benzyloxy, phenylthio or phenoxy;

R<sub>1</sub> is hydroxy or C<sub>1</sub>-C<sub>6</sub>alkoxy;

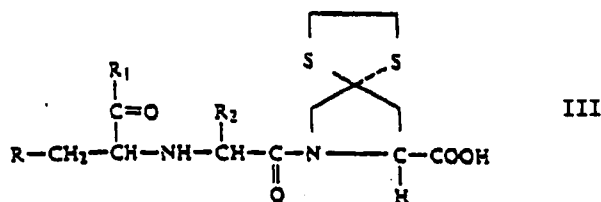
and R<sub>2</sub> is hydrogen, C<sub>1</sub>-C<sub>6</sub>alkyl or C<sub>1</sub>-C<sub>6</sub>aminoalkyl.

In the above formula, preferred compounds are those wherein R is benzyl, R<sub>1</sub> is C<sub>1</sub>-C<sub>6</sub> alkoxy and R<sub>2</sub> is hydrogen, methyl or aminobutyl. More preferred compounds of the above formula are those wherein R is benzyl, R<sub>1</sub> is C<sub>1</sub>-C<sub>6</sub> alkoxy and R<sub>2</sub> is hydrogen or methyl. The even more preferred compounds of the above formula are those wherein R is benzyl, R<sub>1</sub> is ethoxy and R<sub>2</sub> is methyl. The most preferred compound of the above formula is Enalapril having the formula



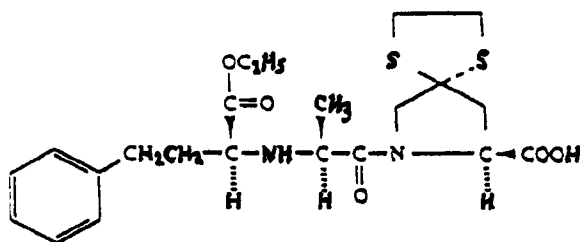
All of the above compounds of formula II are known, having been previously described e.g. in European Patent 12,401. Moreover, their usefulness in treating hypertension as well as methods for their preparation are set forth therein.

A particularly preferred class of ACE inhibitors to which the instant invention would apply are compounds of formula III:



wherein R, R<sub>1</sub> and R<sub>2</sub> have the significances indicated above regarding the compounds of formula II.

The most preferred compound of the above formula is Spirapril having the formula



All of the above compounds of formula III are known, having been previously described e.g. in USP 4,470,972. Moreover, their usefulness in treating hypertension as well as methods for their preparation are set forth therein.

It should be noted that all of the compounds of formulae I, II and III form salts with various inorganic and organic acids and bases, which salts may be prepared by conventional methods. Therefore, it should be understood that all of such salts would also benefit from the use of the select group of hydrochloric acid donors as stabilizers therefor in accordance with the instant invention.

The amount of the active component, e.g. ACE inhibitor, in the pharmaceutical compositions of the instant invention is between 0.5% and 50%, preferably between 0.75% and 25%, e.g. between 1% and 25%, more preferably between 0.75% and 20%, e.g. between 1% and 20%, most preferably between 0.75% and 15%, e.g. between 1% and 15%, based on the total weight of the pharmaceutical composition.

As indicated above, all of the compounds of formulae I, II and III are known and their usefulness in daily

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dosages at which said compounds are employed as well as typical unit dosages of said compounds are well documented in the literature.

Although the pharmaceutical compositions may be in any form, the solid forms are preferred, more preferably tablets, capsules and caplets.

5 In addition to the active components, e.g., an ACE inhibitor, and the stabilizing component, e.g., glycine hydrochloride, the pharmaceutical compositions of the instant invention will typically contain a pharmaceutically acceptable carrier. Generally, they are compounds which do not contain groups which would significantly interfere with either the active component or the stabilizing component. For example, sugars such as lactose, sucrose, mannitol and sorbitol are quite suitable; as are starches such as corn starch and tapioca starch;

10 cellulose and derivatives thereof such as sodium carboxymethyl cellulose, ethyl cellulose and methyl cellulose; calcium phosphates such as dicalcium phosphate; sodium sulfate; and polyvinyl alcohol. Such type compounds are generally present in amounts of between 5% and 90%, preferably between 10% and 80%, based on the total weight of the pharmaceutical composition.

The stabilized compositions of the instant invention may also contain optional ingredients that are normally employed in pharmaceutical formulations, the only qualification being that they must be compatible with the select group of hydrochloric acid donors so as not to interfere with their stabilizing function. Typical optional ingredients include lubricants, e.g., talc, alkaline earth metal stearates such as magnesium stearate and calcium stearate, and hydrogenated vegetable oils such as hydrogenated cottonseed oil; binders such as polyvinylpyrrolidone (povidone) and gelatin; and disintegrants such as microcrystalline cellulose, cross-linked polyvinylpyrrolidone and alginic acid. Other optional ingredients are fillers, water scavengers, buffers, preservatives, anti-oxidants, colorants and flavouring agents. The total amount of the optional ingredients in the stabilized compositions of the instant invention is not critical. In general, the total amount of the optional ingredients is consistent with the amount of the active component, stabilizer and pharmaceutically acceptable carrier, i.e., the total amount will be equivalent to the remainder of the pharmaceutical compositions.

25 The stabilized compositions of the instant invention can be prepared by any of the conventionally employed processing techniques such as the wet granulation process. The technique is preferably chosen to ensure a homogeneous distribution of the active component and a homogeneous distribution of the hydrochloric acid donor over or among the active component particles. Conveniently the hydrochloric acid donor is distributed in a liquid form, e.g. an aqueous solution used as a granulating liquid.

30 Examples of other active components that are contemplated include those with a -NH-CH-CO-N-C-COOH moiety as in the above formula I, II, and III, e.g. the di-acid form of spirapril, spiraprilate. Such compounds include ramipril, perindopril, indolapril, lisinopril, alacepril,trandolapril, benazapril, libenzapril, delapril, and cilazapril.

The following examples are for the purpose of illustration only and are not intended in any way to limit the scope of the instant invention.

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**EXAMPLE 1:**

Below are stabilized compositions in accordance with the instant invention in white tablet form:

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|    | <u>Ingredient</u>       | <u>Amount (mg)</u> |            |
|----|-------------------------|--------------------|------------|
|    |                         | <u>A</u>           | <u>B</u>   |
| 45 | Quinapril hydrochloride | 40.0               | -          |
|    | Enalapril hydrochloride | -                  | 40.0       |
|    | glycine hydrochloride   | 40.0               | 40.0       |
| 50 | lactose                 | 277.5              | 277.5      |
|    | corn starch             | 25.0               | 25.0       |
|    | talc                    | 15.0               | 15.0       |
|    | magnesium stearate      | <u>2.5</u>         | <u>2.5</u> |
| 55 |                         | 400.0              | 400.0      |

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97**EXAMPLE 2:**

The following compositions A-D represent stabilized compositions in accordance with the instant invention in white tablet form whereas composition E does not contain a stabilizer of the instant invention:

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|    | <u>Ingredient</u>               | <u>Amount (mg)</u> |             |             |             |             |
|----|---------------------------------|--------------------|-------------|-------------|-------------|-------------|
|    |                                 | <u>A</u>           | <u>B</u>    | <u>C</u>    | <u>D</u>    | <u>E</u>    |
| 10 | Spirapril hydrochloride         | 3.06               | 3.06        | 3.06        | 3.06        | 3.19        |
|    | lactose, NF                     | 99.94              | 94.74       | 99.94       | 94.74       | 80.21       |
| 15 | starch, NF                      | 19.50              | 19.50       | 19.50       | 19.50       | 12.00       |
|    | povidone, USP                   | 2.60               | 2.60        | 2.60        | 2.60        | 2.00        |
|    | glycine hydrochloride           | -                  | -           | 2.60        | 2.60        | -           |
|    | glutamic acid hydrochloride     | 2.60               | 2.60        | -           | -           | -           |
| 20 | silica gel, NF                  | -                  | 5.20        | -           | 5.20        | 1.90        |
|    | colloidal SiO <sub>2</sub> , NF | 1.30               | 1.30        | 1.30        | 1.30        | 0.10        |
|    | magnesium stearate, NF          | <u>1.00</u>        | <u>1.00</u> | <u>1.00</u> | <u>1.00</u> | <u>0.60</u> |
| 25 | total                           | 130.00             | 130.00      | 130.00      | 130.00      | 100.00      |

**EXAMPLE 3:**

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To demonstrate the effectiveness of the stabilizers of the instant invention against added moisture, the following results were obtained when the compositions of Example 2A-2D were stored for 3 months at 30°C and 75% relative humidity:

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|               | <u>* Assay %</u> | <u>Diketo</u> | <u>Diacid</u> |
|---------------|------------------|---------------|---------------|
| Example 2A    | 99.6             | 0.0           | 0.1           |
| 40 Example 2B | 100.0            | 0.0           | 0.2           |
| Example 2C    | 99.6             | 0.0           | 0.1           |
| Example 2D    | 99.9             | 0.0           | 0.2           |

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\* per cent of original Spirapril hydrochloride content

**EXAMPLE 4:**

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To demonstrate the effectiveness of the stabilizers of the instant invention against an increase in temperature, the following results were obtained when the compositions of Example 2A and 2C were stored at 50°C for varying periods of time. For purposes of comparison, below are the results obtained when the composition of Example 2E was stored at 50°C for three months.

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|    |            | <u>Period (months)</u> | <u>* Assay %</u> | <u>Diketo</u> | <u>Diacid</u> |
|----|------------|------------------------|------------------|---------------|---------------|
| 6  | Example 2A | 1                      | 99.0             | 0.2           | 0.1           |
|    |            | 2                      | 100.8            | 0.6           | 0.3           |
|    |            | 3                      | 99.1             | 0.9           | 0.3           |
| 10 | Example 2C | 1                      | 100.3            | 0.1           | 0.2           |
|    |            | 2                      | 101.3            | 0.8           | 0.2           |
|    |            | 3                      | 98.4             | 1.0           | 0.3           |
| 15 | Example 2E | 3                      | 91.2             | 7.3           | 0.4           |

\* per cent of original Spirapril hydrochloride content

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**EXAMPLE 5:**

The following compositions A and B and D represent stabilized compositions in accordance with the instant invention in colored tablet form whereas composition C contains maleic acid, an acidifier disclosed in the prior art

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|    | <u>Ingredient</u>               | <u>Amount (mg)</u> |             |             |            |
|----|---------------------------------|--------------------|-------------|-------------|------------|
| 30 |                                 | <u>A</u>           | <u>B</u>    | <u>C</u>    | <u>D</u>   |
|    | Spirapril hydrochloride         | 3.06               | 3.06        | 3.06        | 6          |
|    | lactose, NF                     | 96.94              | 96.94       | 96.94       | 99.77      |
| 35 | starch, NF                      | 19.50              | 19.50       | 19.50       | 22.50      |
|    | povidone, USP                   | 2.60               | 2.60        | 2.60        | 3.0        |
|    | alginic acid                    | -                  | -           | -           | 13.0       |
| 40 | glycine hydrochloride           | 2.60               | -           | -           | 3.0        |
|    | glutamic acid hydrochloride     | -                  | 2.60        | -           | -          |
|    | maleic acid                     | -                  | -           | 2.60        | -          |
| 45 | carmine                         | 3.00               | 3.00        | 3.00        | -          |
|    | iron oxide, red                 | -                  | -           | -           | 0.03       |
|    | colloidal SiO <sub>2</sub> , NF | 1.30               | 1.30        | 1.30        | 1.5        |
|    | magnesium stearate, NF          | <u>1.00</u>        | <u>1.00</u> | <u>1.00</u> | <u>1.2</u> |
| 50 |                                 |                    |             |             |            |
|    | total                           | 130.00             | 130.00      | 130.00      | 150.00     |

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**Representative production process**

A batch of 1.6 million tablets of Example 5D is made as follows:

a) Spirapril hydrochloride (4.8 kg), 79.576 kg lactose, starch (18 kg) and povidone (2.4 kg) are sieved separately (typically 1600  $\mu$ m) and mixed together in a high speed mixer.

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b) Iron oxide (0.024 kg) and lactose (0.24 kg) are mixed together and sieved, and added to the mixture obtained in step a).

c) Glycine hydrochloride (2.4 kg) in 13.40 kg of demineralised water is pumped into the mixture obtained in step b), mixed and kneaded until suitable for granulation. The granulate is dried in a fluidized bed drier at 60°C e.g. until a loss on drying of about 2.1% is reached, then broken by sieving (typically 1000 µm). A batch of 107.44 kg results.

d) A second batch of 107.44 kg is produced following steps a), b) and c), and the batches are combined (214.88 kg).

e) In another vessel 20.8 kg alginic acid, 2.4 kg colloidal SiO<sub>2</sub> are mixed together and sieved (typically 1000 µm) and sieved with almost the whole of the granulate obtained in step d).

f) 1.92 kg magnesium stearate and the remaining part of the granulate obtained in step d) are mixed together, sieved (typically 1000 µm) and combined with the mixture in step e). 240 kg of tableting mixture is obtained and this is compressed into tablets.

#### EXAMPLE 6:

To demonstrate the effectiveness of the stabilizers of the instant invention against an increase in temperature in the presence of colorants, the following results were obtained when the carmine colored compositions of Example 5A and 5B were stored at 50°C for three months.

|            | <u>* Assay %</u> | <u>Diketo</u> | <u>Diacid</u> |
|------------|------------------|---------------|---------------|
| Example 5A | 96.3             | 2.7           | **            |
| Example 5B | 96.0             | 1.8           | **            |

\* per cent of original Spirapril hydrochloride content  
\*\* interference from dye

#### EXAMPLE 7:

To show the superiority of the pharmaceutical compositions of the present invention, the following results were obtained when the compositions of Example 5A and 5C were stored at 50°C for varying periods of time:

|            | <u>Period (months)</u> | <u>* Assay %)</u> | <u>Diketo</u> | <u>** Diacid</u> |
|------------|------------------------|-------------------|---------------|------------------|
| Example 5A | 1                      | 98.4              | 1.1           | 1                |
|            | 2                      | 15.2              | 3.1           | 2                |
|            | 3                      | 96.3              | 2.7           | 2                |
| Example 5C | 1                      | 91.6              | 5.1           | 1                |
|            | 2                      | 89.2              | 14.8          | 2                |
|            | 3                      | 84.6              | 10.0          | 2                |

\* per cent of original Spirapril hydrochloride content  
\*\* interference from dye

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100**EXAMPLE 8:**

The following compositions A-D represent stabilized compositions in accordance with the instant invention in white tablet form whereas composition E does not contain a stabilizer of the instant invention

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|    | <u>Ingredient</u>               | <u>Amount (mg)</u> |             |             |             |             |
|----|---------------------------------|--------------------|-------------|-------------|-------------|-------------|
|    |                                 | <u>A</u>           | <u>B</u>    | <u>C</u>    | <u>D</u>    | <u>E</u>    |
| 10 | Spirapril hydrochloride         | 3.0                | 3.3         | 3.3         | 3.3         | 3.3         |
|    | lactose, NF                     | 360.0              | 360.0       | 360.0       | 360.0       | 360.0       |
|    | glycine hydrochloride           | 20.0               | -           | -           | -           | -           |
| 15 | ferric chloride                 | -                  | 20.0        | -           | -           | -           |
|    | betaine hydrochloride           | -                  | -           | 20.0        | -           | -           |
|    | glutamic acid hydrochloride     | -                  | -           | -           | 20.0        | -           |
| 20 | colloidal SiO <sub>2</sub> , NF | 1.0                | 1.0         | 1.0         | 1.0         | 1.0         |
|    | stearic acid, NF                | <u>16.0</u>        | <u>16.0</u> | <u>16.0</u> | <u>16.0</u> | <u>16.0</u> |
|    | total                           | 400.3              | 400.3       | 400.3       | 400.3       | 380.3       |

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**EXAMPLE 9:**

To demonstrate the effectiveness of the pharmaceutical compositions of the instant invention against an increase in temperature and added moisture, the following results were obtained when the compositions of Examples 8A-8D stored for 72 hours. For purposes of comparison, below are the results obtained when the composition of Example 8E was stored for 72 hours under the same conditions.

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|    |            | Temp. (°C) | % Water | *Assay % | Diketo | Diacid |
|----|------------|------------|---------|----------|--------|--------|
| 5  | Example 8A | 0          | 0       | 94       | 0.1    | 0.10   |
|    |            | 65         | 0       | 91       | 0.6    | 0.05   |
|    |            | 65         | 5       | -        | -      | -      |
|    |            | 65         | 10      | 92       | 0.7    | 0.10   |
| 10 |            |            |         |          |        |        |
|    | Example 8B | 0          | 0       | 62       | 0.3    | 0.80   |
|    |            | 65         | 0       | 66       | 0.4    | 0.60   |
|    |            | 65         | 5       | 72       | 0.7    | 1.40   |
| 15 |            | 65         | 10      | 66       | 1.3    | 3.10   |
|    |            |            |         |          |        |        |
|    | Example 8C | 0          | 0       | 94       | 0.1    | 0.40   |
| 20 |            | 65         | 0       | 91       | 4.0    | 0.03   |
|    |            | 65         | 5       | 94       | 0.9    | 0.07   |
|    |            | 65         | 10      | 95       | 0.8    | 0.14   |
| 25 |            |            |         |          |        |        |
|    | Example 8D | 0          | 0       | 95       | 0.2    | 0.03   |
|    |            | 65         | 0       | 91       | 3.6    | 0.03   |
|    |            | 65         | 5       | 97       | 0.4    | 0.10   |
| 30 |            | 65         | 10      | 94       | 0.4    | 0.20   |
|    |            |            |         |          |        |        |
|    | Example 8E | 0          | 0       | 93       | 0.1    | 0.05   |
| 35 |            | 65         | 0       | 87       | 6.0    | 0.04   |
|    |            | 65         | 5       | 79       | 9.0    | 0.20   |
|    |            | 65         | 10      | 65       | 17.0   | 0.30   |

40 **EXAMPLE 10:**

To demonstrate the extended shelf-life of a stabilized composition in accordance with the instant invention, the following results were obtained when the composition of Example 5A was stored for an extended period under various conditions:

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| Period (months) | 30°C |     | 40°C |     | 50°C |     | 30°C/75% RH |     |
|-----------------|------|-----|------|-----|------|-----|-------------|-----|
|                 | DK   | DA  | DK   | DA  | DK   | DA  | DK          | DA  |
| 0               | 0.4  | 0.0 | -    | -   | -    | -   | -           | -   |
| 3               | 0.4  | 0.1 | 1.3  | 0.2 | 2.5  | 0.2 | 0.4         | 0.1 |
| 6               | 0.5  | 0.2 | 1.7  | 0.2 | 3.0  | 0.1 | 0.5         | 0.1 |
| 9               | 0.9  | 0.2 | -    | -   | -    | -   | -           | -   |
| 12              | 1.1  | 0.2 | 2.6  | 0.1 | -    | -   | -           | -   |
| 24              | 1.5  | 0.2 | -    | -   | -    | -   | -           | -   |

DK = diketopiperazine

DA = diacid

**Claims**

1. A pharmaceutical composition comprising an active component liable to degradation and a hydrochloric acid donor.
2. A composition according to claim 1 wherein the active component is an ACE inhibitor.
3. A composition according to claim 1 wherein the active component is spirapril.
4. A composition according to claim 1 wherein the active component is quinapril or enalapril.
5. A composition according to any preceding claim wherein the active component is a hydrochloride acid addition salt.
6. A composition according to any preceding claim wherein the hydrochloric acid donor is an amino acid hydrochloride or a Lewis acid chloride.
7. A composition according to claim 6 wherein the hydrochloric acid donor is glycine hydrochloride, glutamic acid hydrochloride, betaine hydrochloride, alanine hydrochloride, valine hydrochloride, lysine hydrochloride, arginine hydrochloride, aspartic acid hydrochloride, ferric chloride, zinc chloride and aluminium chloride.
8. A composition according to claim 6 wherein the hydrochloric acid donor is glycine hydrochloride.
9. A composition according to any preceding claim wherein the hydrochloric acid donor is present in an amount between 1% and 25%, based on the total weight of the composition.
10. A composition according to any preceding claim wherein the weight ratio of active component to hydrochloric acid donor is from about 2.5:1 to about 1:7.
11. A composition according to any preceding claim in the form of a tablet.
12. A stabilised pharmaceutical composition substantially as hereinbefore described with reference to any one of the examples.

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13. A process for the production of a stabilized pharmaceutical composition as defined in any one of claims 1 to 12 which comprises working up an active component liable to degradation with a hydrochloric acid donor.
- 6 14. A method of stabilizing a pharmaceutical composition as defined in any one of claims 1 to 12 containing an active component liable to degradation comprising incorporating therein a stabilizing effective amount of a hydrochloric acid donor.
- 10 15. Use of a hydrochloric acid donor to stabilize an active component liable to degradation in a pharmaceutical composition as defined in any one of claims 1 to 12.
16. Use of a hydrochloric acid donor as stabilizing agent in the manufacture of a pharmaceutical composition containing an active component liable to degradation as defined in any one of claims 1 to 12.

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**United States Patent** [19]  
**Vincent et al.**

[11] **Patent Number:** 4,914,214  
[45] **Date of Patent:** Apr. 3, 1990

[54] **PROCESS FOR THE INDUSTRIAL  
SYNTHESIS OF PERINDOPRIL**

[75] **Inventors:** Michel Vincent, Bagneux; Jean  
Baliarda, Anthony; Bernard  
Marchand, Checy; Georges Remond,  
Versailles, all of France

[73] **Assignee:** Adir Et Cie, Neuilly-sur-Seine,  
France

[21] **Appl. No.:** 245,446

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[30] **Foreign Application Priority Data**

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[51] **Int. Cl.<sup>4</sup>** ..... C07D 209/18

[52] **U.S. Cl.** ..... 548/492

[58] **Field of Search** ..... 548/492

*Primary Examiner*—David B. Springer  
*Attorney, Agent, or Firm*—Gordon W. Hueschen

[57] **ABSTRACT**

Process for the industrial synthesis of perindopril, in which (2S,3aS,7aS)-2-carboxyperhydroindole is condensed with N-[(S)-1-carbethoxybutyl]-(S)-alanine after protection of the carboxyl group, the product resulting from the condensation being then subjected to deprotection of the carboxyl carried by the heterocyclic ring.

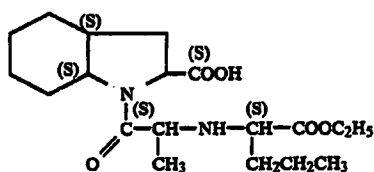
The (2S,3aS,7aS)-2-carboxyperhydroindole and N-[(S)-1-carbethoxybutyl]-(S)-alanine are themselves obtained in excellent conditions from 2-carboxyindole and from L-alanine respectively, both available on an industrial scale.

**5 Claims, No Drawings**

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# PROCESS FOR THE INDUSTRIAL SYNTHESIS OF PERINDOPRIL

The present invention relates to a process for the industrial synthesis of perindopril of formula (I):



or (2S,3aS,7aS)-1-{2-[1-(ethoxycarbonyl)-(S)-butylamine]-(S)-propionyl}octahydroindole-2-carboxylic acid and its tert-butylamine salt.

The compound of formula (I) and its addition salts and, more particularly, its tert-butylamine salt have interesting pharmacological properties. They exert an inhibiting activity on certain enzymes, such as carboxypeptidases, enkephalinases or kininase II. In particular, they inhibit the conversion of the angiotensin I decapeptide to angiotensin II octapeptide, which is responsible in certain cases for arterial hypertension, by acting on the conversion enzyme.

The use of these compounds in therapeutics makes it possible, therefore, to reduce or even to suppress the activity of these enzymes, which are responsible for the hypertensive disorder or for cardiac insufficiency.

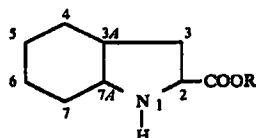
In particular, the compound of formula (I) is distinguished from other conversion enzyme inhibitors by the intensity and the duration of its action.

The compound of formula (I), its preparation and its use in therapeutics have been described in European Patent No. 0,049,658.

However, the industrial preparation of a derivative such as the compound of formula (I) requires an indepth study of all the reaction stages, of the choice of the starting materials, of the reactants, and of the solvents which make it possible to obtain optimum yields.

The Applicant Company has found a process for the synthesis of the compound of formula (I), in which these conditions have been combined through the use of a set of particularly advantageous methods and processes.

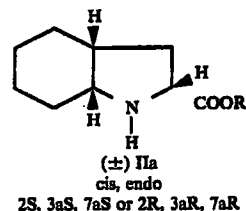
In particular, one of the starting materials which can be employed for the preparation of the compounds of formula (I) is 2-carboxyperhydroindole, described in European Patent Application 0,037,231, or one of its esters of formula (II):



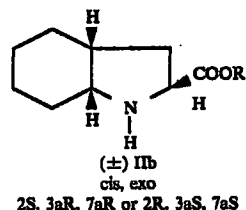
where R denotes a hydrogen atom or a lower alkyl or benzyl group.

The compound of formula (II) exists in the form of four racemic pairs:

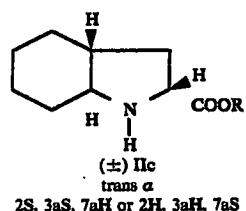
the two cis IIa and IIb epimers,  
the two trans IIc and IId epimers,



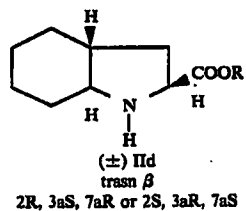
2S, 3aS, 7aS or 2R, 3aR, 7aR



2S, 3aR, 7aR or 2R, 3aS, 7aS



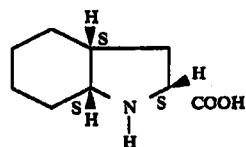
2S, 3aS, 7aH or 2H, 3aH, 7aS



2R, 3aS, 7aR or 2S, 3aR, 7aS

The preparation of a compound of formula (II) can be carried out by means of well-known methods of the prior art (EP 0,037,231, EP 0,084,164, EP 0,115,345, EP 0,173,199, EP 0,132,580).

However, the isomer which is specifically employed in the synthesis of the compound of formula (I), on account of its structure, is (2S,3aS,7aS)-2-carboxyperhydroindole of formula (IIaS):



(IIaS)

The preparation of (2S,3aS,7aS)-2-carboxyperhydroindole can be carried out according to the methods described in the prior art (EP 0,037,231, EP 0,115,345, EP 0,173,199, EP 0,132,580).

The starting material employed by some of these is 2-carboxyperhydroindole which has the advantage of being a starting material which is readily available and relatively inexpensive (EP 0,037,231), which is subjected to catalytic reduction over rhodium to give a mixture of the two cis endo isomers of (2S,3aS,7aS) and (2R,3aR,7aR) configuration respectively.

However, the separation of the (2S,3aS,7aS) isomer which can be used in the synthesis of the compound of

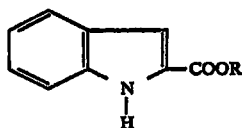
formula (I) from the (2R,3aR,7aR) isomer generally requires the use of methods which are particularly arduous to employ.

Thus, to perform this separation of the (2S,3aS,7aS) and (2R,3aR,7aR) (cis, endo racemic) isomers, Patent No. 0,037,231 employs many stages requiring the synthesis of the N-benzoyl compound, fractional crystallization of the salt formed by the diastereoisomer with S- $\alpha$ -phenylethylamine, the liberation of the two N-benzoylated SSS and RRR compounds, and then the removal of the benzoyl group, followed by a pass through an ion exchange column and a recrystallization.

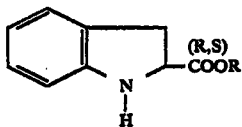
For this same separation, European Patent Application No. 0,115,345 employs several stages requiring the esterification of the carboxylic acid group with benzyl alcohol, the conversion of the amino ester to a salt with (S)-N-benzylloxycarbonylphenylalanine, the separation of the S,S,S isomer by fractional crystallization, and the liberation of the amino group, optionally followed by the liberation of the carboxylic acid group.

To synthesize the compound of formula (I) on an industrial scale, the Applicant Company employs an original process for the synthesis of (2S,3aS,7aS)-2-carboxyperhydroindole, which also offers the advantage of employing 2-carboxyindole as starting material, but which does not offer the disadvantage of this arduous separation of the two (2S,3aS,7aS) and (2R,3aR,7aR) isomers, because, in a first step, 2-carboxyindole is reduced to 2-carboxyindoline, to give a mixture of 2R and 2S carboxyindolines which are easily separated in a single stage, by fractional crystallization. The 2S isomer is then subjected to catalytic hydrogenation to lead stereoselectively to (2S,3aS,7aS)-2-carboxyperhydroindole.

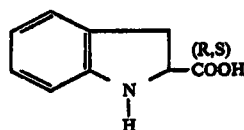
More particularly, the starting material employed in the industrial synthesis of (2S,3aS,7aS)-2-carboxyperhydroindole, employed by the Applicant Company for the industrial synthesis of the compound of formula (I), is 2-carboxyindole or one of its esters of formula (III):



in which R denotes a hydrogen atom or a lower alkyl group, which is subjected to reduction by a process such as the use of the tin/hydrochloric acid couple, preferably in a lower aliphatic alcohol medium, preferably at ambient temperature, to (R,S)-2-carboxyindoline or to one of its esters of formula (IV):



in which R has the same meaning as in formula (III), which, when R=H, is the 2-carboxyindoline of formula (V), which, when R is other than H, is converted by alkaline hydrolysis into 2-carboxyindoline of formula (V):



consisting, in fact, of a mixture of two isomers, according to whether the carboxyl-bearing carbon is:

in the R configuration (R isomer),

in the S configuration (S isomer),

from which mixture the S isomer is isolated by adding the said mixture to a solution of (+)- $\alpha$ -methylbenzylamine in a lower aliphatic alcohol, to obtain a precipitate of the salt formed by (S)-2-carboxyindoline with  $\alpha$ -methylbenzylamine,

which, after filtration, is dissolved in water, the solution obtained being then acidified to permit the liberation of (S)-2-carboxyindoline,

which, after filtration and washing, is subjected to catalytic hydrogenation,

under a hydrogen pressure of between 10 and 150 bars, preferably chosen between 20 and 60 bars,

with heating to a temperature of between 30° and 100° C., preferably between 40° and 80° C.,

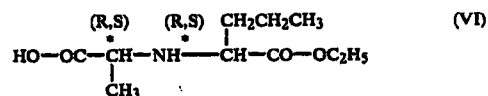
the catalyst being chosen from rhodium, palladium, platinum or nickel, mixed with a support such as charcoal, so as to make it possible to obtain a maximum proportion of (2S,3aS,7aS)-2-carboxyperhydroindole,

the latter being separated from the (2S,3aR,7aR) isomer obtained in a low proportion by a single crystallization

from a polar solvent strictly chosen from lower aliphatic alcohol, acetonitrile, ethyl acetate and dioxane,

by itself or mixed with water, or mixed with each other and with water, provided that the mixture obtained forms a single phase.

The second starting material employed for the synthesis of the compound of formula (I) according to the invention is the compound of formula (VI):



The compound of formula (VI) exists in the form of four stereoisomers which may be denoted as (R,R) (R,S), (S,R) or (S,S) according to the configuration of the two so-called asymmetric carbons.

Now, the most active compound of formula (I) is that in the case of which the two carbons in the side chain both have the S configuration.

This is the reason why the process for the industrial synthesis of the compound of formula (I) according to the invention concerns exclusively the industrial synthesis of the compound of formula (VI), in which the two asymmetric carbons both have the S configuration.

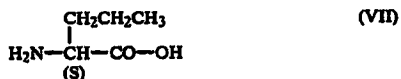
No process for the specific industrial synthesis of the compound of formula (VI) has been described. European Patent Application No. 0,117,488, which is very general and which employs  $\alpha$ -carboxylated trifluoromethanesulfonates, is known. However, the stereochemistry of both starting materials must be strictly chosen in order to obtain the desired diastereoisomer of the product of formula (I).

It is furthermore known to a person skilled in the art that, as a very general rule, to permit the separation of diastereoisomers which are obtained in the course of syntheses where the stereochemistry of the starting materials is not fixed beforehand, traditional techniques such as fractional crystallization or chromatography on a silica column are resorted to.

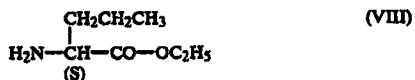
In the case of the industrial synthesis of the compound of the formula (I), the Applicant Company employs a process for the industrial synthesis of the compound of formula (VI) which is of great interest because, on the one hand, it is particularly simple to implement and, on the other hand, it makes possible, by means of a judicious choice of the reactants (catalysts and solvents) employed to obtain the (S,S) diastereoisomer directly in yields which are highly advantageous on an industrial scale.

Furthermore, the synthesis according to the process of the invention offers the advantage of employing inexpensive compounds as starting materials, and this is of importance on an industrial scale.

More particularly, the starting material employed in the process employed by the Applicant Company for the industrial synthesis of the compound of formula (I) is a compound of a natural amino acid, of formula (VII):



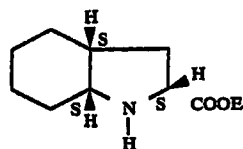
in which the asymmetric carbon has the S configuration, since it is well known to a person skilled in the art that the carboxyl-bearing carbon in natural amino acids has the S configuration (with the exception of cysteine), which is treated, in the presence of an acidic esterification catalyst, and preferably of thionyl chloride, with ethanol, which is industrially available at a low price, to give the ester of formula (VIII):



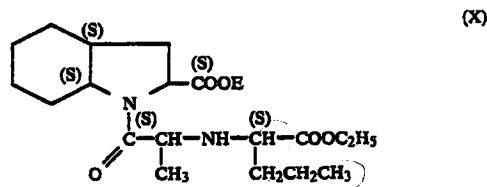
which is condensed under catalytic hydrogenation, under a hydrogen pressure of between 10 and 150 bars, preferably chosen between 20 and 60 bars, preferably at ambient temperature, the catalyst being chosen from rhodium, palladium, platinum or nickel, mixed with a support such as charcoal, a catalyst carefully chosen so as to direct the selectivity of the reaction, thus making it possible to obtain a maximum proportion of the (S,S) diastereoisomer, with pyruvic acid  $\text{CH}_3-\text{CO}-\text{COOH}$ , a natural, inexpensive and industrially available product, to lead directly, after a single crystallization from a solvent carefully chosen from lower aliphatic alcohol, acetonitrile, ethyl acetate and dioxane, by itself or mixed with water, or mixed with each other and with water, provided that the mixture obtained forms a single phase, cooling and filtration, solely to the (S,S) diastereoisomer of the compound of formula (VI).

The final stage of the industrial synthesis of the compound of formula (I) according to the invention consists in the condensation of the compound of formula (IIaS), whose carboxylic acid group has been protected beforehand by esterification with an alcohol EOH, where E preferably denotes a linear or branched lower alkyl or

benzyl group in the presence of an acidic esterification catalyst, to give an ester of (2S,3aS,7aS)-2-carboxy-perhydroindole of formula (IX):



which is condensed directly with the compound of formula (VI) in an alkaline medium in the presence of a catalyst for peptide synthesis such as dicyclohexylcarbodiimide, to lead to the derivative of formula (X):



in which E has the same meaning as in formula (IX), which is subjected to deprotection of the carboxylic group of the heterocyclic ring, to lead the compound of formula (I), in the form of a base, which is dissolved in a solvent chosen from lower aliphatic alcohol, acetonitrile, ethyl acetate, dioxane by itself or mixed with each other, before the addition of tert-butylamine, the salt thus obtained being crystallized by heating the reaction mixture, filtering hot, cooling and finally filtering off.

The following example illustrates the invention, but does not limit it in any way.

#### EXAMPLE

Industrial Synthesis of the tert-Butylamine Salt of (2S,3aS,7aS)-1-2-[1-(ethoxycarbonyl)-(S)-butylamino]-(S)-propionyl]-octahydroindole-2-carboxylic acid

Stage 1: (2S,3aS,7aS)-2-Carboxy-octahydroindole

Stage 1a: 2-Ethoxycarbonylindole

Heat 5 kg of 2-carboxyindole suspended in ethanol in the presence of sulfuric acid to boiling for 8 hours. Evaporate.

Evaporate off the ethyl acetate, take up the crystalline mass with hexane. After filtering off and drying, 5.3 kg of crystals are obtained.

Melting point: 123°-125° C.

Microanalysis: Calculated: C % 69.83; H % 5.86; N % 7.40; Found: C % 69.56; H % 5.74; N % 7.30.

Spectrometry in the infrared: 2150  $\text{cm}^{-1}$  (NH); 1680  $\text{cm}^{-1}$  (carboxylic acid).

Stage 1B: (R,S)-2-Ethoxycarbonylindoline

Suspend, in a reactor, 10 kg of 2-ethoxycarbonylindoline obtained previously in 110 liters of hydrochloric ethanol. Next, add 20 kg of granulated tin. Keep stirring for approximately 2 days at ambient temperature.

Evaporate off the ethanol, take up the residue with water and add 110 liters of toluene. Stir for approximately 20 minutes. Alkalify with aqueous ammonia.

Separate off the aqueous phase and extract once again with 150 liters of toluene.

Combine the toluene phases and wash them with water. Separate off the toluene phases, filter. Remove the water by distilling the water-toluene azeotrope. Cool and pass through a stream of anhydrous HCl gas.

Cool. Evaporate down and wash with pure toluene.

Weight obtained: 10.11 kg.

Yield: 84%.

Thin layer chromatography: Solvent: toluene: 10; ethyl acetate: 5; Support: Merck silica 60 F 254; Developer: UV;  $R_f$ : 0.55.

#### Stage 1C: (R,S)-2-Carboxyindoline

2.15 kg of (R,S)-2-ethoxycarbonylindoline dissolved in ethanol are saponified with 12.5 liters of N sodium hydroxide with stirring for 24 hours. After washing the alkaline solution, neutralize with concentrated hydrochloric acid. After filtering off, washing and drying, 1.57 kg of white crystals of the expected product are obtained.

Yield: 86%.

Melting point: 188°-189° C.

Spectrometry in the infrared:  $\text{NH}_2^+$ : 2500-2000  $\text{cm}^{-1}$ ;  $\text{COO}^-$ : 1620  $\text{cm}^{-1}$ .

#### Stage 1D: (S)-2-Carboxyindoline

6.05 kg of (R,S)-2-carboxyindoline are added to a solution of 4.49 kg of (+)- $\alpha$ -methylbenzylamine in anhydrous ethanol. A white precipitated product is obtained which, after filtering off, is digested in refluxing isopropanol. After cooling, the solid is filtered off and washed with a little isopropanol; the white crystals obtained are dried: 3.68 kg.

Rotatory power:  $[\alpha]_D^{25} = -5.3$  ( $c=1\%$  ethanol).

(S)-2-Carboxyindoline is prepared in a quantitative yield by dissolving 1 kg of the above salt in 5 liters of water and neutralizing with an aqueous hydrochloric acid solution. This precipitate is filtered off, washed with water and dried.

#### Stage 1E: (2S,3aS,7aS)-2-Carboxyoctahydroindole

Place 25 kg of (S)-2-carboxyindoline, obtained previously, in 110 liters of methanol in a vessel. Keep stirred. Charge the rhodium (5% dry) catalyst into a mixer.

Start up the stirring in a hydrogenator, charge the methanolic suspension of (S)-2-carboxyindoline by passing it through the mixer and rinse the assembly with water. Heat to 60° C. and pressurize with hydrogen (30 bars).

Filter off the catalyst on a single-plate filter. Collect the hydroalcoholic liquors in a reactor and evaporate the methanol off under vacuum.

After concentrating, charge approximately 300 kg of dioxane. Heat to boiling and add water until a solution is obtained. Allow to cool. Filter off and dry. 22.3 kg of crystals are obtained.

Yield: 86.1%.

#### Stage 2: N-[(S)-1-Carbethoxybutyl]-(S)-alanine

##### Stage 2A: Ethyl L-norvalinate hydrochloride

Place 35 kg of L-norvaline in approximately 300 kg of denatured ethanol in a reactor. Introduce approximately 60 kg of thionyl chloride, slowly and gradually.

After stirring for a quarter of an hour, heat to reflux for 3 hours and then evaporate off the ethanol under vacuum.

Take up the residue with 300 liters of cyclohexane and heat to boiling. Allow to cool, filter, wash with cyclohexane and dry. 52.9 kg of ethyl L-norvalinate hydrochloride are obtained, that is a 97.6% yield.

The product thus obtained is employed as such in the next stage.

#### Stage 2B: N-[(S)-1-carbethoxybutyl]-(S)-alanine

Place 45 kg of ethyl N-norvalinate hydrochloride obtained in the preceding stage and approximately 110 liters of water in a vessel equipped with a stirrer.

Alkalify, then pour 23 kg of pyruvic acid very gradually into the solution obtained previously and stir the reaction mixture for 30 minutes.

Place an aqueous suspension of charcoal containing 5% palladium and the alkaline solution of ethyl L-norvalinate obtained previously in a hydrogenation apparatus.

Hydrogenate under pressure (30 bars) at ambient temperature for approximately one day.

Filter under vacuum and evaporate the filtrate under reduced pressure, filter off and dry. Treat the residue obtained with ethanol; remove the insoluble material, consisting of sodium chloride, by filtration and rinse it with ethanol. Combine the ethanolic solutions; evaporate off the ethanol under reduced pressure and crystallize the residue from acetonitrile.

34.3 kg of N-[(S)-1-carbethoxybutyl]-(S)-alanine are obtained, that is a 63.9% yield.

#### Stage 3: tert-Butylamine salt of (2S,3aS,7aS)-1-[2-[1-ethoxycarbonyl]-(S)-butylamino]-(S)-propionyl]octahydroindole-2-carboxylic acid

##### Stage 3A: para-Toluenesulfonate of the benzyl ester of (2S,3aS,7aS)-2-carboxyoctahydroindole

In a 30-liter reactor, reflux 12.5 kg of (2S,3aS,7aS)-2-carboxyperhydroindole, 50 kg of para-toluenesulfonic acid and 14.2 kg of benzyl alcohol and 38.4 kg of toluene, removing the water formed with the aid of a continuous separator. When no more water separates out, cool, filter off the precipitate formed, and dry.

Yield: 91.3%.

##### Stage 3B: Benzyl ester of (2S,3aS,7aS)-1-[2-[1-(ethoxycarbonyl]-(S)-butylamino]-(S)-propionyl]octahydroindole-2-carboxylic acid

Add approximately 3.5 kg of triethylamine to a suspension of approximately 5 kg of para-toluenesulfonate of the benzyl ester of (2S,3aS,7aS)-2-carboxyoctahydroindole in approximately 60 kg of ethyl acetate, followed by approximately 6 kg of 1-hydroxybenzotriazole, approximately 7.5 kg of the N-[(S)-1-carbethoxybutyl]-(S)-alanine obtained in stage 2 and approximately 7.0 kg of dicyclohexylcarbodiimide.

Stir, cooling slightly for approximately 3 hours, then filter off the dicyclohexylurea formed by filtration and wash the organic phase with water. The dried organic phase is evaporated to dryness.

Yield: 92.3%.

#### Stage 3C:

##### (2S,3aS,7aS)-1-[2-[1-(Ethoxycarbonyl]-(S)-butylamino]-(S)-propionyl]octahydroindole-2-carboxylic acid

Dissolve, in a hydrogenator, 14 kg of benzyl ester of the (2S,3aS,7aS)-1-[2-[1-(ethoxycarbonyl]-(S)-

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butylamino]-(S)-propionyl]octahydroindole-2-carboxylic acid obtained in the preceding stage in cyclohexane.

Add the charcoal containing 5% palladium and approximately 50 liters of water. Hydrogenate at ordinary temperature and pressure until the theoretical volume of hydrogen has been absorbed. Filter, wash the insoluble material with cyclohexane, separate off the organic phase and wash the aqueous phase again with cyclohexane. Isolate the product from the aqueous phase by freeze-drying.

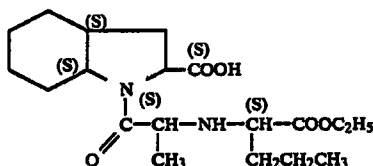
Stage 3D: tert-Butylamine salt of (2S,3aS,7aS)-1-[2-[1-(ethoxycarbonyl)-(S)-butylamino]-(S)-propionyl]octahydroindole-2-carboxylic acid

Place in a reactor approximately 140 liters of ethyl acetate and 10 kg of (2S,3aS,7aS)-1-[2-[1-(ethoxycarbonyl)-(S)-butylamino]-(S)-propionyl]octahydroindole-2-carboxylic acid obtained previously. Add gradually approximately 2.20 kg of tert-butylamine, heat to reflux until all has dissolved; filter. Cool, filter off and dry.

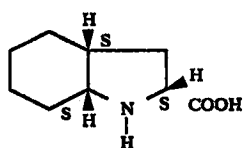
Yield: 95%.

I claim:

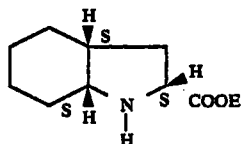
1. Process for the synthesis of the tert-butylamine salt of (2S,3aS,7aS)-1-[2-[1-(ethoxycarbonyl)-(S)-butylamino]-(S)-propionyl]octahydroindole-2-carboxylic acid of formula (I):



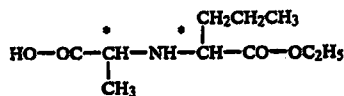
wherein (2S,3aS,7aS)-2-carboxyperhydroindole of formula (IIaS)



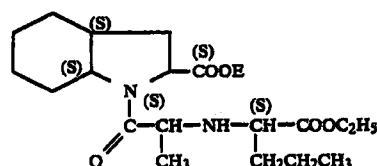
is protected by esterification with an alcohol EOH, where E denotes a linear or branched lower alkyl group or the benzyl group, in the presence of an acidic esterification catalyst to give an ester of (2S,3aS,7aS)-2-carboxyperhydroindole of formula (IX):



is condensed with to the (S,S) diastereoisomer of N-[(S)-1-carbethoxybutyl]-(S)-alanine of formula (VI):



in an alkaline medium in the presence of a catalyst for peptide synthesis such as dicyclohexylcarbodiimide in the presence of 1-hydroxybenzotriazole to lead to the compound of formula (X):



in which E has the same meaning as in formula (IX), which is subjected to deprotection of the carboxylic group of the heterocyclic ring, to lead to the compound of formula (I) in the form of a base,

which is dissolved in a solvent chosen from lower aliphatic alcohol, acetonitrile, ethyl acetate, dioxane by itself or mixed with each other, before the addition of tert-butylamine, the salt thus obtained being crystallized by heating the reaction mixture, filtering hot, cooling and finally filtering off.

2. A process for the synthesis of the tert-butylamine salt of (2S,3aS,7aS)-1-[2-[1-(ethoxycarbonyl)-(S)-butylamino]-(S)-propionyl]octahydroindole-2-carboxylic acid, as claimed in claim 1, wherein the carboxylic acid group of (2S,3aS,7aS)-2-carboxyperhydroindole preceding the condensation of the latter with N-[(S)-1-carbethoxybutyl]-(S)-alanine of formula (VIII), is protected by reaction with benzyl alcohol.

3. A process for the synthesis of the tert-butylamine salt of (2S,3aS,7aS)-1-[2-[1-(ethoxycarbonyl)-(S)-butylamino]-(S)-propionyl]octahydroindole-2-carboxylic acid, as claimed in one of claim 1 or 2, wherein the carboxylic acid group of (2S,3aS,7aS)-2-carboxyperhydroindole preceding the condensation of the latter with N-[(S)-1-carbethoxybutyl]-(S)-alanine of formula (VIII) is protected by reaction with benzyl alcohol, using para-toluenesulfonic acid as catalyst.

4. A process for the synthesis of the tert-butylamine salt of (2S,3aS,7aS)-1-[2-[1-(ethoxycarbonyl)-(S)-butylamino]-(S)-propionyl]octahydroindole-2-carboxylic acid, as claimed in one of claim 1, 2, or 3, wherein the deprotection of the carboxylic acid group of (2S,3aS,7aS)-2-carboxyperhydroindole following the condensation of the latter with N-[(S)-1-carbethoxybutyl]-(S)-alanine of formula (VIII) is performed by catalytic hydrogenation.

5. A process for the synthesis of the tert-butylamine salt of (2S,3aS,7aS)-1-[2-[1-(ethoxycarbonyl)-(S)-butylamino]-(S)-propionyl]octahydroindole-2-carboxylic acid, as claimed in one of claim 1, 2, 3, or 4, wherein the deprotection of the carboxylic acid group of (2S,3aS,7aS)-2-carboxyperhydroindole following the condensation of the latter with N-[(S)-1-carbethoxybutyl]-(S)-alanine of formula (VIII) is performed by catalytic hydrogenation at ambient temperature and pressure, in the presence of palladized charcoal.

\* \* \* \* \*

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## The Haemodynamic and Humoral Effects of Treatment for One Month with the Angiotensin Converting Enzyme Inhibitor Perindopril in Salt Replete Hypertensive Patients

K. R. Lees and J. L. Reid

University Department of Materia Medica, Stobhill General Hospital, Glasgow, U. K.

**Summary.** We have studied the effects of treatment for one month with perindopril, 4 or 8 mg once daily, in seven hypertensive patients. Blood pressure was lowered from 164/93 mm Hg to 145/84 mm Hg by 4 mg of perindopril and after one month remained at 142/82 mm Hg. Neither postural hypotension nor tachycardia occurred.

Inhibition of plasma angiotensin converting enzyme (ACE) lasting for over 24 h was achieved and there was a significant increase in plasma renin activity (PRA).

Maximum plasma concentrations of the active metabolite of perindopril, S-9780, were detected four h after oral administration.

After treatment for one month there was evidence of reduced sensitivity of plasma ACE to the action of the inhibitor. The plasma concentration of S-9780 required to produce 50% inhibition of plasma ACE rose from  $2.4 \text{ ng} \cdot \text{ml}^{-1}$  following the first dose to  $5.5 \text{ ng} \cdot \text{ml}^{-1}$  after one month.

**Key words:** perindopril, hypertension; angiotensin converting enzyme inhibition, renin-angiotensin system

Perindopril (S-9490-3; Servier Laboratories, Neuilly sur Seine, France) is a new, orally-active inhibitor of angiotensin converting enzyme (ACE). It has a non-thiol structure (Fig. 1) and although itself relatively inactive it is rapidly hydrolysed in vivo to the active diacid metabolite, S-9780. This compound is a potent and long-lasting inhibitor of ACE in animals [1] and in man [2, 3]. We have reported that perindopril is well tolerated in man in doses of up to 16 mg once daily for periods of up to one week [2, 3]. Hypertensive patients might be expected to show a greater response to perindopril, and we have therefore ex-

amined the tolerability of perindopril and studied its biochemical and haemodynamic effects in patients with essential hypertension.

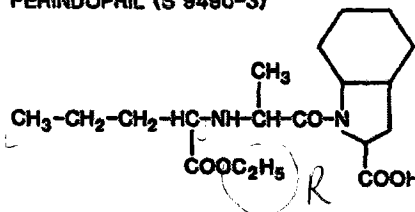
### Methods

#### Patients

Male and post-menopausal female patients, aged 18-65 y, with essential hypertension (BP 145/95 to 200/115, Phase V supine, untreated), but no other significant disease, were considered for entry.

All patients gave written consent to a protocol approved by the local ethics committee. The patients underwent full history, physical examination, and pre-study tests which included routine urinalysis, haematology, serum biochemistry, and an electrocardiogram. Seven patients took part and all completed

PERINDOPRIL (S 9490-3)



(S 9780)

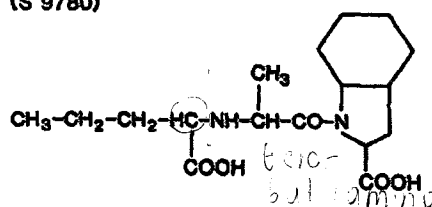


Fig. 1. Chemical structures of perindopril and its active diacid metabolite, S-9780

# L-Arginine Abrogates Salt-sensitive Hypertension in Dahl/Rapp Rats

Pei Yan Chen and Paul W. Sanders

Nephrology Research and Training Center and Division of Nephrology, Department of Medicine, University of Alabama at Birmingham, and Veterans Administration Medical Center, Birmingham, Alabama 35294

## Abstract

This study examined the contribution of nitric oxide (NO) to the susceptibility or resistance to the hypertensive effects of high sodium chloride (8.0% NaCl) intake in young Dahl/Rapp salt-sensitive (SS/Jr) and salt-resistant (SR/Jr) rats. Using  $N^G$ -monomethyl-L-arginine (L-NMMA) as a probe for NO production in vivo, we found that increasing dietary sodium chloride increased NO activity in salt-resistant rats, but not in salt-sensitive rats. Exogenous L-arginine, the substrate for NO synthesis, decreased blood pressure to normotensive levels in salt-sensitive rats made hypertensive for 2 wk from 8.0% NaCl chow. D-Arginine had no effect on blood pressure of these rats and L-arginine did not change blood pressure of salt-resistant rats. Intraperitoneal injections of L-arginine and its precursor, L-citrulline, and oral L-arginine, but not D-arginine, prevented the increase in blood pressure in salt-sensitive rats on the high salt chow over 2 wk of observation. In contrast, L-arginine did not alter the development of hypertension in spontaneously hypertensive rats. Mean urinary cGMP levels were higher in salt-sensitive rats on oral L-arginine than salt-sensitive rats on D-arginine. Infusion of L-NMMA acutely decreased, whereas intravenous L-arginine rapidly increased, urinary cGMP in both groups. L-Arginine and L-citrulline increased production of NO and prevented salt-sensitive hypertension in Dahl/Rapp rats. (*J. Clin. Invest.* 1991; 88:1559-1567.) Key words: endothelium-derived nitric oxide • endothelium-derived relaxing factor •  $N^G$ -monomethyl-L-arginine • nitric oxide synthase • urinary cyclic guanosine monophosphate • spontaneously hypertensive rats

## Introduction

With the growing interest in the vascular endothelium as an endocrine organ that secretes vasodilatory and vasoconstrictor hormones (1, 2), recent studies have shown that endothelial cells secrete an important paracrine hormone, endothelium-

derived nitric oxide (3). Nitric oxide (NO)<sup>1</sup> has a short biologic half-life (4) and produces vasodilation by relaxation of smooth muscle cells of several arterial resistance vessels, including the renal vasculature (5), by increasing cGMP through activation of cytosolic guanylate cyclase (4, 6). The enzyme involved in the production of NO has been termed nitric oxide (NO) synthase and is stereoselective, utilizing L-arginine but not D-arginine as the substrate to produce nitric oxide (4, 7, 8). NO synthase is competitively inhibited by  $N^G$ -monomethyl-L-arginine (L-NMMA) (4, 7, 9, 10) or by other L-arginine molecules that have substitutions on the guanidino nitrogen atoms (4, 10). L-NMMA serves as a chemical probe of NO production in vivo and in vitro. Intravenous infusion of L-NMMA produces rapid increases in blood pressure in humans (11), rats (5, 12), rabbits (9), and guinea pigs (2); this increase in blood pressure is reversed with intravenous infusion of L-arginine. Thus, production of NO is an important determinant of resting arteriolar tone.

The pioneering work of Lewis K. Dahl produced from the Sprague-Dawley line two strains of rats that were either susceptible (S) or resistant (R) to the hypertensive effects of a high salt (8.0% NaCl) diet (13, 14). The Dahl S and R rats were inbred by John P. Rapp for 20 generations to yield two strains that were each homozygous at almost 100% of all genetic loci, thus fixing the characteristics of the strains (14). These Dahl/Rapp rats were either salt-sensitive (SS/Jr) or salt-resistant (SR/Jr). On an 8.0% NaCl diet, the SS/Jr rats rapidly and uniformly developed hypertension and died by 12 wk of age (14). Thus, the Dahl/Rapp strains provide excellent models to investigate salt-sensitive hypertension and salt resistance.

We postulated that NO participates in the blood pressure response to changes in dietary sodium chloride intake. The purpose of this study was to examine the role of NO in the prevention and production of salt-sensitive hypertension in the SS/Jr and SR/Jr Dahl/Rapp rats.

## Methods

### Protocols

**Series 1 (L-NMMA protocol).** To examine the effect of dietary NaCl on the response to L-NMMA and L-arginine in vivo, male, 21-d-old, salt-sensitive (SS/Jr) and salt-resistant (SR/Jr) Dahl/Rapp rats (Harlan Sprague-Dawley, Indianapolis, IN) were maintained on tap water and either 0.3% NaCl or 8.0% NaCl chow (both from Dyets, Inc., Bethlehem, PA) ad libitum for 2 wk. The diets were identical in composition except for the NaCl content. They were then anesthetized with ethyl, 1-methylpropylthiobarbiturate (Inactin; BYK Gulden, Hamburg, FRG), 100 mg/kg body wt, i.p., and placed on a servo-controlled heated table.

Portions of the material in this manuscript were presented at the 1990 meetings of the American Society of Nephrology and the Southern Section of the American Federation for Clinical Research and were published in abstract form (1990. *J. Am. Soc. Nephrol.* 1:439; and 1990. *Clin. Res.* 38:960A).

Address correspondence and reprint requests to Paul W. Sanders, M.D., Division of Nephrology/Department of Medicine, Sixth Floor, Zeigler Building, University of Alabama at Birmingham, Birmingham, AL 35294.

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1. Abbreviations used in this paper: GFR, inulin clearance; L-NMMA,  $N^G$ -monomethyl-L-arginine; MAP, mean arterial pressure; NO, nitric oxide; SR/Jr, Dahl/Rapp salt-resistant rat; SS/Jr, Dahl/Rapp salt-sensitive rat.

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Rectal temperature, which was maintained at 37°C, was monitored with a telethermometer (Yellow Springs Instrument Co., Yellow Springs, OH). Tracheostomy was performed with polyethylene PE-240 tubing, followed by catheterization of the right external jugular vein with PE-50 tubing for infusion of Ringer-bicarbonate with 8% polyfructosan (Inutest; Laevosan-Gesellschaft, Linz, Austria) at 1.2 ml/100 g body wt/h after a 1-ml bolus; this infusion was maintained throughout the experiment. The left external jugular vein was then cannulated with PE-50 tubing for administration of drugs during the experiment. The right femoral artery was cannulated to record arterial blood pressure using a unigraph (model ICT-1H; Gilson Medical Electronics, Middleton, WI) and to sample blood. The bladder was catheterized through a suprapubic route for urine collection. After a 60-min equilibration period, arterial blood samples were collected for determination of hematocrit and plasma concentrations of inulin, sodium, and chloride. Two 30-min inulin clearances were obtained, then *N*<sup>ω</sup>-monomethyl-L-arginine (L-NMMA) (Sigma Chemical Co., St. Louis, MO) was given intravenously as a bolus, 50 mg/kg body wt, followed by continuous infusion at 500 μg/kg body wt/min. This dose of L-NMMA was similar to that used by Tolins et al. (12). 10 min after infusion began a 30-min inulin clearance was performed. Mean arterial pressure (MAP) was recorded 30 min after the bolus was given. At the completion of the clearance, the infusion of L-NMMA was stopped and L-arginine (Sigma Chemical Co.), 300 mg/kg body wt, was given as an intravenous bolus. 10 min later another 30-min inulin clearance was obtained and MAP 30 min after the intravenous injection of L-arginine was recorded.

In a separate experiment, this same protocol was repeated using 21-d-old male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) on either 0.3 or 8.0% NaCl chow for 2 wk. Results were compared between these two groups.

**Series 2 (intravenous L-arginine treatment).** To examine the effect of intravenous L-arginine and D-arginine on salt-sensitive hypertension, 21-d-old SS/Jr and SR/Jr rats were maintained on 8.0% NaCl chow and tap water ad libitum for 2 wk. They were then anesthetized and surgically prepared as described above. After the 1-h equilibration period, a 30-min inulin clearance was obtained, then the rats received either D-arginine or L-arginine, 300 mg/kg body wt, as an intravenous bolus. The inulin clearance was repeated and MAP was determined 30 min after the bolus. At the completion of the inulin clearance, the rats were given another intravenous bolus of either D-arginine or L-arginine, 300 mg/kg body wt. A 30-min inulin clearance was performed and the MAP was determined 30 min after the second bolus.

**Series 3 (intrapertoneal L-arginine and L-citrulline treatment).** To study the effect of i.p. L-arginine on the prevention of salt-sensitive hypertension, 21-d-old SS/Jr rats were maintained on 0.3% NaCl chow and tap water ad libitum for 2 wk. 2 d before study, the rats were anesthetized with ether and the left femoral artery was catheterized with polyethylene tubing, which was tunneled subcutaneously to the nape of the neck. The animals were allowed to recover for 1 d. The diet was then changed to 8.0% NaCl chow and they were given daily i.p. injections of D-arginine, 250 mg/kg body wt, or L-arginine, 250, 100, or 25 mg/kg body wt. Approximately 2 h before each injection, MAP was recorded in these awake, unrestrained rats for 5 d. The rats given the higher amounts of L-arginine and D-arginine were continued for 2 wk. They were subsequently anesthetized with Inactin and prepared surgically as described in series 1. After the 1-h equilibration period, a 30-min inulin clearance was obtained, then L-NMMA was given intravenously as a bolus, 50 mg/kg body wt, followed by continuous infusion at 500 μg/kg body wt per min. 30 min after the bolus, MAP was recorded and the experiment was terminated.

In similar experiments, L-citrulline, 250 mg/kg body wt, or vehicle was injected i.p. daily for 5 d. After the sixth day, these rats were anesthetized and examined using the protocol described above.

In a separate study, 16 spontaneously hypertensive rats (SHR) (Charles River Breeding Laboratories, Inc., Wilmington, MA) 6 wk of age were placed on 8% NaCl chow and tap water ad libitum for 2 wk. During this time, they received i.p. injections of L-arginine or D-arginine,

250 mg/kg body wt per day. Systolic blood pressures were determined using a tail cuff attached to a blood pressure analyzer (IITC, Inc., Woodland Hills, CA) twice weekly in these rats while in a conscious, prewarmed, and restrained state. At the end of the 2-wk period, the rats were anesthetized with ether and a catheter was inserted into the left femoral artery and tunneled subcutaneously to the nape of the neck. Two rats died inexplicably during the recovery period from the ether anesthesia. The rats were allowed to recover for 1 d, then MAP was recorded in conscious and unrestrained rats. The rats were then anesthetized with Inactin, prepared surgically and the protocol described above was repeated. In addition, urine samples from each clearance period were collected in 0.1 mM 3-isobutyl-1-methylxanthine (Sigma Chemical Co.) and immediately frozen and stored at -20°C. The samples were processed simultaneously for determination of cGMP.

**Series 4 (oral L-arginine treatment).** This protocol was similar to the one described in series 3. 2 wk after beginning the 0.3% NaCl diet, the left femoral artery was catheterized for recording awake blood pressure as described above. The animals were allowed to recover for 1 d, then the diet was then changed to 8.0% NaCl chow and they were given either D-arginine or L-arginine, 1.25 g/liter, to drink ad libitum for 2 wk. MAP was recorded each morning in these awake, unrestrained rats. The rats were weighed daily and the amount of fluid intake recorded. After 2 wk, the rats were anesthetized with Inactin and prepared surgically as described in the series 1. After the 1-h equilibration period, a 30-min inulin clearance was obtained, then L-NMMA was given intravenously as a bolus, 50 mg/kg body wt, followed by continuous infusion at 500 μg/kg body wt per min. 10 min after infusion began a 30-min inulin clearance was performed. MAP was recorded 30 min after the bolus was given. At the completion of the clearance, the infusion of L-NMMA was stopped and L-arginine, 300 mg/kg body wt, was given as an intravenous bolus. MAP was recorded 30 min after the intravenous injection of L-arginine and the experiment was terminated.

In these experiments, the urine samples from each period were collected in 0.1 mM 3-isobutyl-1-methylxanthine (Sigma Chemical Co.) and immediately frozen and stored at -20°C. The samples were processed simultaneously for determination of cGMP.

### Analytical techniques

Plasma and urine sodium concentrations were determined with a flame photometer (model IL-943; Instrumentation Laboratories, Inc., Lexington, MA). Plasma and urine chloride concentrations were determined amperometrically (Buchler digital chloridometer; Buchler Instruments, Inc., Fort Lee, NJ). Concentration of inulin in plasma and urine was determined by the anthrone colorimetric method, which has been standardized in our laboratory (15). Clearance of inulin (GFR) was calculated from these values and was factored by body weight. Measurement of cGMP was accomplished with a cGMP ELISA kit (Cayman Chemical Co., Ann Arbor, MI).

### Statistical analysis

All values were expressed as mean±SEM. Comparisons between two groups and within a group were analyzed for statistical significance by unpaired and paired *t* tests, respectively. Comparisons among more than two groups were analyzed for statistical significance by analysis of variance and the Scheffe *F*-test using statistical software (Statview 512+; Abacus Concepts, Inc., Berkeley, CA). Significance was set at the 5% level.

### Results

**Series 1 (L-NMMA protocol) (Table I).** At the time of study, mean age and body weight of the four groups were 37±0 d and 162±4 g and did not differ among the four groups (data not shown). After surgical preparation and the equilibration period, baseline urinary excretion rates of sodium (35±12 vs. 22±4 nEq/min per 100 g body wt) and chloride (66±9 vs.

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Table I. Effects of L-NMMA and L-Arginine on MAP and GFR in Anesthetized, 37-d-old SS/Jr and SR/Jr Rats and Sprague-Dawley Rats after 14 d on the Indicated NaCl Diet

| Group | Dietary NaCl | n | Baseline data |                                        | L-NMMA                   |                  |                                        | L-Arginine               |                                        |
|-------|--------------|---|---------------|----------------------------------------|--------------------------|------------------|----------------------------------------|--------------------------|----------------------------------------|
|       |              |   | MAP           | GFR                                    | MAP                      | % change in MAP  | GFR                                    | MAP                      | GFR                                    |
|       |              |   | mmHg          | $\mu\text{l/min}/100\text{ g body wt}$ | mmHg                     | %                | $\mu\text{l/min}/100\text{ g body wt}$ | mmHg                     | $\mu\text{l/min}/100\text{ g body wt}$ |
| SS/Jr | 0.3          | 6 | 106 $\pm$ 3   | 1044 $\pm$ 46                          | 130 $\pm$ 5 <sup>§</sup> | +22.2 $\pm$ 1.5  | 1059 $\pm$ 50                          | 107 $\pm$ 4              | 914 $\pm$ 65                           |
| SR/Jr | 0.3          | 8 | 111 $\pm$ 2   | 1034 $\pm$ 88                          | 134 $\pm$ 2 <sup>§</sup> | +20.5 $\pm$ 1.3  | 1050 $\pm$ 106                         | 108 $\pm$ 3              | 1065 $\pm$ 30                          |
| SS/Jr | 8.0          | 6 | 138 $\pm$ 1*  | 1089 $\pm$ 48                          | 168 $\pm$ 1 <sup>§</sup> | +21.4 $\pm$ 0.7  | 1037 $\pm$ 41                          | 125 $\pm$ 2 <sup>§</sup> | 990 $\pm$ 106                          |
| SR/Jr | 8.0          | 6 | 114 $\pm$ 2   | 1330 $\pm$ 37*                         | 149 $\pm$ 2 <sup>§</sup> | +31.1 $\pm$ 0.7* | 834 $\pm$ 128 <sup>§</sup>             | 113 $\pm$ 3              | 1084 $\pm$ 80                          |
| P     |              |   | 0.0001        | 0.008                                  | 0.0001                   | 0.0001           |                                        | 0.001                    |                                        |
| SD    | 0.3          | 5 | 114 $\pm$ 3   | 521 $\pm$ 69                           | 141 $\pm$ 4 <sup>§</sup> | +23.9 $\pm$ 1.0  | 1115 $\pm$ 294                         | 111 $\pm$ 4              | 933 $\pm$ 221                          |
| SD    | 8.0          | 5 | 113 $\pm$ 2   | 670 $\pm$ 68                           | 157 $\pm$ 5 <sup>§</sup> | +38.9 $\pm$ 4.0  | 1115 $\pm$ 143 <sup>§</sup>            | 118 $\pm$ 4              | 958 $\pm$ 162                          |
| P     |              |   |               |                                        | 0.03                     | 0.006            |                                        |                          |                                        |

SD, Sprague-Dawley. \* Statistically different value among the four groups of Dahl/Rapp rats. <sup>§</sup>  $P = 0.0001$  vs. SR/Jr group on 0.3% NaCl diet. <sup>§</sup>  $P < 0.05$  vs. the baseline values in the same group.

86 $\pm$ 13 nEq/min per 100 g body wt) did not differ between SR/Jr and SS/Jr rats on the low-salt chow. The SR/Jr and SS/Jr groups on the high-salt chow had baseline urinary excretion rates of sodium (498 $\pm$ 152 vs. 942 $\pm$ 179 nEq/min per 100 g body wt) and chloride (759 $\pm$ 187 vs. 1,303 $\pm$ 225 nEq/min per 100 g body wt) that did not differ and were higher ( $P = 0.0004$ ) than the two groups on the low-salt chow. SR/Jr and SS/Jr rats on the 0.3% NaCl diet and SR/Jr rats on 8.0% NaCl chow had similar MAPs, whereas MAP of SS/Jr rats on the 8.0% NaCl diet was higher ( $P = 0.0001$ ) than the other three groups (Table I). GFR was higher ( $P = 0.008$ ) in the SR/Jr rats on the 8.0% NaCl diet, compared with the other three groups. L-NMMA infusion produced a rapid (within 2 min) and sustained increase ( $P < 0.05$ ) in MAP in all four groups; but compared with the increase in MAP in the SR/Jr rats on the low-salt diet, MAP increased to a greater degree ( $P = 0.0001$ ) in the SR/Jr group on the high-salt diet. The 31.1 $\pm$ 0.7% increase in MAP in the SR/Jr group on the high-salt diet during L-NMMA infusion was greater ( $P = 0.0001$ ) than the other three groups (Table I). Infusion of L-NMMA did not change GFR of the SR/Jr rats on

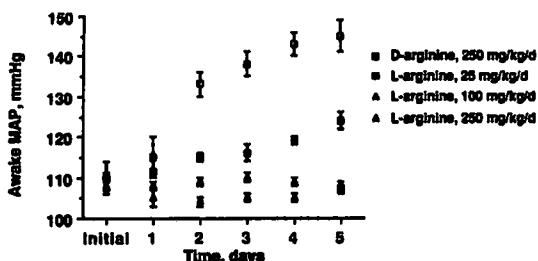
the low-salt chow, but decreased GFR 37.6 $\pm$ 8.6% in SR/Jr rats on the high-salt chow. L-NMMA infusion did not change GFR and increased MAP in proportional increments (22.2 $\pm$ 1.5% vs. 21.4 $\pm$ 0.7%) in SS/Jr rats on the two diets. With L-arginine infusion, final MAP was higher ( $P = 0.001$ ) in the SS/Jr rats on the 8.0% NaCl chow than the other three groups, but was lower ( $P < 0.05$ ) than the baseline MAP of this high-salt group.

Mean body weights of the low-salt and the high-salt groups of Sprague-Dawley rats at the time of study were 151 $\pm$ 4 and 144 $\pm$ 3 g. After surgical preparation and the equilibration period, baseline urinary excretion rates of sodium (38 $\pm$ 5 vs. 329 $\pm$ 124 nEq/min per 100 g body wt) and chloride (54 $\pm$ 4 vs. 400 $\pm$ 169 nEq/min per 100 g body wt) reflected differences in the dietary sodium chloride intake of the low- and high-salt groups. Baseline MAP did not differ between the two groups (Table I). Hypertension did not develop in any of the five rats after 2 wk on the high-salt chow. With infusion of L-NMMA, MAP rose further ( $P = 0.03$ ) in the rats on the high-salt chow. Mean GFR at baseline did not differ between these groups and both increased during infusion of L-NMMA to comparable lev-

Table II. Effects of L-Arginine and D-Arginine on MAP and GFR in Anesthetized, 38-d-old SS/Jr and SR/Jr Rats after 14 d on the 8.0% NaCl Diet

| Group      | n | Baseline     |                                        | Arginine dose            |                  |                                        | 600 mg/kg                |                  |
|------------|---|--------------|----------------------------------------|--------------------------|------------------|----------------------------------------|--------------------------|------------------|
|            |   | MAP          | GFR                                    | MAP                      | % change in MAP  | GFR                                    | MAP                      | % change in MAP  |
|            |   | mmHg         | $\mu\text{l/min}/100\text{ g body wt}$ | mmHg                     | %                | $\mu\text{l/min}/100\text{ g body wt}$ | mmHg                     | %                |
| D-Arginine |   |              |                                        |                          |                  |                                        |                          |                  |
| SS/Jr      | 6 | 140 $\pm$ 3  | 1007 $\pm$ 34                          | 146 $\pm$ 5*             | +0.6 $\pm$ 0.6   | 1016 $\pm$ 44                          | 142 $\pm$ 5*             | -2.1 $\pm$ 1.7   |
| L-Arginine |   |              |                                        |                          |                  |                                        |                          |                  |
| SS/Jr      | 6 | 145 $\pm$ 4  | 1035 $\pm$ 43                          | 117 $\pm$ 6 <sup>§</sup> | -16.6 $\pm$ 2.9* | 996 $\pm$ 48                           | 110 $\pm$ 4 <sup>§</sup> | -21.1 $\pm$ 2.8* |
| SR/Jr      | 7 | 118 $\pm$ 4* | 1086 $\pm$ 42                          | 117 $\pm$ 4              | -0.2 $\pm$ 0.7   | 1111 $\pm$ 30                          | 114 $\pm$ 4              | -2.6 $\pm$ 0.8   |
| P          |   | 0.0002       |                                        | 0.0008                   | 0.0001           |                                        | 0.0001                   | 0.0001           |

\* Statistically different value among the three groups. <sup>§</sup>  $P < 0.05$  compared with the baseline values.



**Figure 1.** Effects of daily i.p. injections of D-arginine, 250 mg/kg body wt, and L-arginine, 250, 100, and 25 mg/kg body wt, on MAP of awake unrestrained SS/Jr rats on the high-salt diet. At baseline, MAP of the four groups of rats did not differ. In the rats given D-arginine, a rapid and sustained increase in MAP from  $110 \pm 4$  to  $145 \pm 4$  mmHg occurred over the 5 d of observation. This increase in blood pressure became significant ( $P = 0.0001$ ) from the other three groups by the second day of observation. MAP remained unchanged in the groups receiving L-arginine, 250 mg/kg body wt per d ( $108 \pm 1$  to  $108 \pm 1$  mmHg), and L-arginine, 100 mg/kg body wt per d ( $108 \pm 1$  to  $107 \pm 1$  mmHg), and did not differ. An intermediate response was seen in the group given L-arginine, 25 mg/kg body wt per d, with MAP increasing from  $110 \pm 1$  to  $124 \pm 2$  mmHg by the fifth day. By day four, the blood pressure response of this group receiving the lowest dose of L-arginine differed ( $P = 0.0001$ ) from the other two groups given the higher doses of L-arginine.

els (Table I). Because of the variability of the response, however, the increase in GFR of the low-salt group did not reach statistical significance ( $P = 0.08$ ).

**Series 2 (intravenous L-arginine treatment) (Table II).** These rats had been fed the 8.0% NaCl chow for 14 d before study. Mean age of the rats at the time of study was  $38 \pm 0$  d and was not different among the three groups (data not shown). After surgical preparation and equilibration, baseline urinary excretion rates of sodium (in nEq/min per 100 g body wt) were  $776 \pm 103$  (SS/Jr-D-arginine),  $1,468 \pm 458$  (SS/Jr-L-arginine), and  $789 \pm 173$  (SR/Jr-L-arginine) and did not differ among the three groups. Baseline urinary excretion rates of chloride (in nEq/min per 100 g body wt) were  $1,363 \pm 256$  (SS/Jr-D-arginine),  $1,869 \pm 516$  (SS/Jr-L-arginine), and  $1,169 \pm 249$  (SR/Jr-L-arginine) and did not differ among the three groups. As expected, on the high-salt diet MAP was higher ( $P = 0.0002$ ) in the SS/Jr rats than the SR/Jr rats; average GFRs were similar in all three groups (Table II). Infusion of D-arginine or L-arginine produced rapid, transient decreases in MAP in both SS/Jr and SR/Jr rats; within 5 min, MAP returned to baseline in the SR/Jr rats treated with L-arginine and in SS/Jr rats treated with D-arginine. After 30 min, the L-arginine infusion produced no

net change in MAP in the SR/Jr rats, but decreased MAP  $21.1 \pm 2.8\%$  in the SS/Jr rats; thus, MAP of SS/Jr rats was lowered to values comparable to the SR/Jr strain. The D-arginine infusion did not change MAP of the SS/Jr rats. After the second injection of L-arginine, GFR fell slightly in the SS/Jr rats from  $1,035 \pm 43$  to  $902 \pm 73$   $\mu\text{L}/\text{min}$  per 100 g body wt ( $P < 0.05$ ).

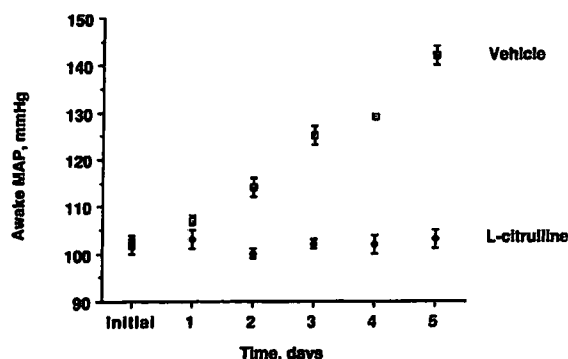
**Series 3 (intraperitoneal L-arginine and L-citrulline treatment).** Daily i.p. injections of L-arginine, 100 and 250 mg/kg body wt per d, prevented the rise in awake MAP in SS/Jr rats on the 8.0% NaCl diet, but i.p. injections of D-arginine had no effect on the increase in MAP, which developed rapidly and was sustained over the 5 d of observation (Fig. 1). L-Arginine, 25 mg/kg body wt per d, i.p., partially prevented the increase in MAP in these rats (Fig. 1). At 2 wk, mean body weights of the rats receiving D-arginine and L-arginine, 250 mg/kg body wt per d, were comparable (Table III). MAP under anesthesia remained elevated ( $152 \pm 4$  mmHg) in the D-arginine group, but was  $118 \pm 2$  mmHg in the L-arginine group. GFRs did not differ between these groups. With L-NMMA infusion, MAP rose to similar levels in both groups, but the percent increase ( $44.7 \pm 3.2\%$ ) in MAP was greater ( $P = 0.0004$ ) in the L-arginine group (Table III). The  $18.0 \pm 1.3\%$  increase in MAP in the D-arginine group from L-NMMA infusion did not differ ( $P = 0.1445$ ) from the responses that occurred in the SS/Jr rats on either diet and the SR/Jr rats on the low-salt diet in series 1 (Table I).

Daily i.p. injections of L-citrulline, 250 mg/kg body wt, prevented the increase in blood pressure in SS/Jr rats on the high-salt chow (Fig. 2). When studied on the sixth day of treatment, mean body weights and GFRs did not differ (Table IV). MAP under anesthesia remained elevated ( $148 \pm 2$  mmHg) in the vehicle-treated rats, but was  $106 \pm 1$  mmHg in the L-citrulline group. The percent increase in MAP in response to infusion of L-NMMA (Table IV) was comparable to that seen in the L-arginine-treated animals (Tables I and III).

In the spontaneously hypertensive rats treated i.p. with L-arginine or D-arginine, mean body weights (Table V) did not differ. Initial mean systolic blood pressures ( $146 \pm 7$  vs.  $148 \pm 6$  mmHg;  $P = 0.8777$ ) also did not differ. Comparable increases in mean systolic blood pressures in the two groups treated with L-arginine or D-arginine were found at the end of the first ( $164 \pm 5$  vs.  $155 \pm 7$  mmHg;  $P = 0.2546$ ) and second weeks ( $186 \pm 2$  vs.  $189 \pm 2$  mmHg;  $P = 0.2106$ ). Awake MAP ( $165 \pm 4$  vs.  $163 \pm 5$  mmHg;  $P = 0.7804$ ) of the two groups at the end of the 2-wk period did not differ. Anesthetized MAP and GFR and the responses to L-NMMA and L-arginine were comparable (Table V). Urinary [cGMP] at baseline ( $25.7 \pm 4.6$  vs.  $32.0 \pm 5.7$  pmol/ml GFR per 100 g body wt;  $P = 0.4043$ ), during infusion of L-NMMA ( $43.9 \pm 14.6$  vs.  $45.2 \pm 11.4$  pmol/ml GFR

**Table III.** Data from SS/Jr Rats after 2 wk on 8% NaCl Chow and Daily i.p. Injections of Either L-Arginine or D-Arginine

| Group      | n | Body wt<br>g | Baseline data |                                                | L-NMMA      |                   |
|------------|---|--------------|---------------|------------------------------------------------|-------------|-------------------|
|            |   |              | MAP           | GFR                                            | MAP         | % increase in MAP |
|            |   |              | mmHg          | $\mu\text{L}/\text{min}/100 \text{ g body wt}$ | mmHg        | %                 |
| L-Arginine | 7 | $221 \pm 7$  | $118 \pm 2$   | $993 \pm 54$                                   | $171 \pm 3$ | $44.7 \pm 3.2$    |
| D-Arginine | 6 | $224 \pm 13$ | $152 \pm 4$   | $942 \pm 29$                                   | $179 \pm 5$ | $18.0 \pm 1.3$    |
| P          |   |              | 0.0001        |                                                |             | 0.0001            |



**Figure 2.** Effect of daily i.p. injections of L-citrulline, 250 mg/kg body wt, or vehicle on MAP of awake unrestrained SS/Jr rats on the high-salt diet. In the rats given vehicle, a rapid and sustained increase in MAP from  $102 \pm 2$  to  $142 \pm 2$  mmHg occurred over the 5 d of observation. Over the same interval, MAP remained unchanged ( $102 \pm 1$  to  $103 \pm 2$  mmHg) in the L-citrulline group. Differences between the blood pressures of the two groups became significant ( $P = 0.0001$ ) by the second day of treatment.

per 100 g body wt;  $P = 0.9496$ ), and after intravenous bolus of L-arginine ( $25.1 \pm 6.7$  vs.  $21.7 \pm 5.3$  pmol/ml GFR per 100 g body wt;  $P = 0.7059$ ) did not differ between the two groups. Within each group, L-NMMA had a small effect on MAP (Table V) and no effect on urinary [cGMP]; L-arginine did not reduce MAP below baseline and did not change urinary [cGMP].

**Series 4 (oral L-arginine treatment).** SS/Jr rats on the D-arginine drink developed increases in MAP on the high-salt diet, whereas those rats given the L-arginine drink remained normotensive (Fig. 3). One of the rats on D-arginine died 10 d into the study. At 2 wk, mean body weights (Table VI) and fluid intake ( $84 \pm 4$  vs.  $85 \pm 4$  ml/d) did not differ. Mean GFR and MAP were greater in the D-arginine group (Table VI). With infusion of L-NMMA, blood pressure increased in both groups. Again, the percent increase in MAP was greater ( $P = 0.0001$ ) in the L-arginine group, whereas the  $17.1 \pm 2.2\%$  increase that occurred in the D-arginine group was similar to the increases seen in the SS/Jr groups in series 1 (Table I). L-Arginine infusion rapidly corrected the blood pressure in the L-arginine group and lowered ( $P < 0.05$ ) MAP  $\sim 30$  mmHg below baseline MAP in the D-arginine treated rats (Table VI).

Mean baseline urinary cGMP concentration was higher ( $P = 0.03$ ) in the L-arginine group than the D-arginine group (Table VII). With infusion of L-NMMA, urinary cGMP rapidly

decreased in both groups to comparable levels as blood pressure increased. With cessation of the L-NMMA infusion and the single intravenous bolus of L-arginine, urinary cGMP increased in both groups, and was higher ( $P = 0.03$ ) in the L-arginine group.

## Discussion

Since the original description of endothelium-derived relaxing factor by Furchgott and Zawadzki (16), several laboratories reported that this paracrine hormone was NO or a combination of NO and a labile nitroso precursor of NO (1–4, 8). NO, a gas that has a very short half-life, has been difficult to measure (4). Most assays for NO have not quantitated NO directly but instead used indirect means such as cGMP generation (4, 6, 12, 17), bioassay that examines changes in relaxation of aortic smooth muscle strips (4, 8, 18), or the effects of inhibitors of NO production (2, 4, 5, 7, 9, 11, 12, 17). NO has been shown to be synthesized by endothelial cells in vivo and in culture from L-arginine, but not from D-arginine (1, 2, 4, 7–9); synthesis of NO was stereospecifically inhibited by *N*<sup>G</sup>-monomethyl-L-arginine (L-NMMA) (1, 2, 4, 7, 9, 17, 18). Because L-NMMA had no direct constrictor or dilator effects on vascular smooth muscle, this drug increased blood pressure by abolishing endothelium-derived vasodilation through inhibition of NO production (9, 18). Our current studies utilized this information to demonstrate several new findings in blood pressure regulation in the Dahl/Rapp salt-sensitive (SS/Jr) and salt-resistant (SR/Jr) strains of rat. Using L-NMMA as a probe of NO activity, we showed that NO was present in SS/Jr, SR/Jr and Sprague-Dawley rats on the 0.3% NaCl diet and was a determinant of resting arteriolar tone even on this low-salt diet (Table I). These data were consistent with previous reports demonstrating a basal release in resistance vessels of NO, which maintained baseline arteriolar tone and blood pressure in humans, rats, rabbits, and guinea pigs (2, 5, 9, 11, 12). L-NMMA infusion also produced a significantly greater and reversible increase in blood pressure in SR/Jr and Sprague-Dawley rats on the 8.0% NaCl diet, compared with the corresponding strains of rats on the 0.3% NaCl chow (Table I). In contrast, L-NMMA infusion produced proportionate increases in MAP in SS/Jr rats on both the low- and high-salt chow (Fig. 4). Finally, provision of L-arginine and its precursor, L-citrulline, but not D-arginine, rapidly normalized blood pressure in hypertensive SS/Jr rats fed the high-NaCl diet for 2 wk (Table II) and, importantly, prevented the development of hypertension caused by the high-salt diet during observation for 2 wk (Tables III, IV, and VI). In addition, baseline mean urinary cGMP level was higher in normotensive SS/Jr

**Table IV.** Data from SS/Jr Rats after 6 d on 8% NaCl Chow and Daily i.p. Injections of Either L-Citrulline or Vehicle

| Group        | n | Body wt<br>g | Baseline data |                             | L-NMMA      |                      |
|--------------|---|--------------|---------------|-----------------------------|-------------|----------------------|
|              |   |              | MAP<br>mmHg   | GFR<br>μl/min/100 g body wt | MAP<br>mmHg | % Increase<br>in MAP |
| L-Citrulline | 6 | 194 ± 7      | 106 ± 1       | 918 ± 47                    | 149 ± 3     | 42.6 ± 2.7           |
| Vehicle      | 6 | 197 ± 4      | 148 ± 2       | 746 ± 65                    | 172 ± 5     | 16.4 ± 1.4           |
| P            |   |              | 0.0001        | 0.06                        |             | 0.0001               |

Table V. Effects of L-NMMA and L-Arginine on Spontaneously Hypertensive Rats after 2 wk on the 8% NaCl Chow and Given i.p. Either L-Arginine or D-Arginine

| Group      | n | Body wt<br>g | Baseline Data |                                        | L-NMMA      |                 |                                        | L-Arginine  |
|------------|---|--------------|---------------|----------------------------------------|-------------|-----------------|----------------------------------------|-------------|
|            |   |              | MAP           | GFR                                    | MAP         | Change in MAP   | GFR                                    | MAP         |
|            |   |              | mmHg          | $\mu\text{l/min}/100\text{ g body wt}$ | mmHg        | %               | $\mu\text{l/min}/100\text{ g body wt}$ | mmHg        |
| L-Arginine | 8 | 155 $\pm$ 6  | 165 $\pm$ 3   | 923 $\pm$ 84                           | 181 $\pm$ 3 | +9.9 $\pm$ 2.6  | 1165 $\pm$ 104                         | 166 $\pm$ 2 |
| D-Arginine | 6 | 168 $\pm$ 6  | 160 $\pm$ 3   | 902 $\pm$ 115                          | 180 $\pm$ 3 | +13.0 $\pm$ 3.5 | 1016 $\pm$ 147                         | 162 $\pm$ 6 |

No statistically significant differences were found between the two groups.

rats treated for 2 wk with the L-arginine drink, compared to the hypertensive rats that received the D-arginine drink. Urinary cGMP decreased while blood pressure increased acutely with infusion of L-NMMA; urinary cGMP increased while blood pressure fell rapidly after stopping this infusion and administering a single intravenous bolus of L-arginine (Fig. 5). Tolins and associates (12) demonstrated a significant correlation between endothelium-dependent changes in blood pressure and urinary cyclic GMP in Sprague-Dawley rats treated with acetylcholine, which stimulates production of endothelium-derived relaxing factor. Thus, although direct measurements of nitric oxide were not performed in our study, indirect assays that included determination of changes in blood pressure and urinary cGMP, which are the functional results of NO production, and use of a competitive inhibitor of NO synthesis demonstrated an important role of dietary sodium chloride in augmenting NO activity in the SR/Jr strain. NO activity did not increase in the SS/Jr rats in response to increases in dietary sodium chloride and hypertension developed unless provided L-arginine or L-citrulline, a precursor of L-arginine production in endothelial cells (19).

With L-NMMA infusion, GFR fell despite an increase in blood pressure in the SR/Jr rats on the 8.0%, but not the 0.3%, NaCl diet. L-NMMA infusion did not change GFR in the SS/Jr rats on either diet and increased GFR in the Sprague-Dawley rats on the high-salt diet (Table I). These data suggested that unlike the age-matched Sprague-Dawley and SS/Jr rats, in the SR/Jr rats during high NaCl intake NO was an important factor maintaining GFR, thus facilitating excretion of NaCl and

perhaps partially explaining their marked resistance to high dietary sodium chloride intake. Glomerular endothelial cells in culture have been shown to release NO which increased cGMP concentrations in cultured mesangial cells (20). In separate studies, renal vascular resistance of Sprague-Dawley rats increased with a decrease in NO (5). Thus, the different response of GFR to L-NMMA in the SR/Jr rats on the high-salt diet may have reflected an enhanced sensitivity of glomerular mesangial cells, which serve as one control of glomerular ultrafiltration (21), or renal vasculature to decreased NO activity.

Our data suggested an important role of NO in salt-sensitive hypertension in the SS/Jr rats. Unlike the SR/Jr and Sprague-Dawley rats, the high-salt diet did not enhance the effects of L-NMMA on blood pressure in the SS/Jr rats (Fig. 4 and Table I); we interpreted these data to suggest that NO production was not increased in these animals in response to the increase in dietary NaCl. However, another potential explanation for this apparent lack of modulation of NO production was chronic hypertension, which has been shown to decrease endothelium-dependent relaxation of aortic rings obtained from several models of hypertension including the Dahl rat; this defect, which presumably was secondary to diminished production of endothelium-derived NO, corrected when hypertension was reversed (22). Dohi and associates (23) demonstrated a decrease in endothelium-dependent inhibition of norepinephrine-induced contractions in dissected mesenteric arteries of 16–20-wk-old normotensive Wistar-Kyoto rats, compared with arteries from rats 8 wk of age; hypertension contributed to this inhibition in the 16–20-wk-old spontaneously hypertensive rats. These findings underscore the importance of examining NO activity in young rats that have been hypertensive for a short duration. Our SS/Jr rats were 5–7 wk old and had been fed the 8.0% NaCl chow for only 2 wk, so that they were overtly hypertensive for a relatively short period of time. Intravenous boluses of L-arginine, the substrate for NO synthesis (1, 2, 4, 7–9), acutely decreased blood pressure of hypertensive SS/Jr rats to levels similar to the SR/Jr rats (Tables I and II). In contrast, L-arginine did not lower blood pressure in spontaneously hypertensive rats, which had blood pressure of elevation and duration comparable to the SS/Jr rats (Table V). Thus, although the absence of augmented NO activity could have been secondary to the effects of hypertension itself, the resistance vessels of the hypertensive SS/Jr rats responded rapidly and appropriately to the addition of the substrate for endothelium-dependent relaxation and allowed blood pressure to return to normotensive levels. This vasodilatory effect of L-arginine occurred selectively in SS/Jr rats and not in spontaneously hypertensive rats.

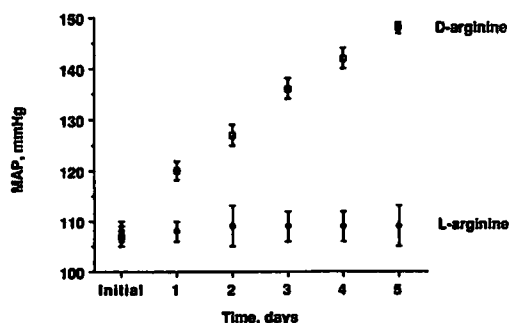


Figure 3. Effect of oral L-arginine on MAP of awake unrestrained SS/Jr rats on the high-salt diet. MAP increased from 108 $\pm$ 2 to 148 $\pm$ 1 mmHg in the D-arginine group, but did not change (107 $\pm$ 2 to 109 $\pm$ 4 mmHg) in the L-arginine group. Average blood pressures of the two groups differed ( $P < 0.005$ ) by the second day of treatment.

Table VI. Effects of L-NMMA and L-Arginine on Anesthetized SS/Jr Rats after 2 wk on 8% NaCl Chow and Oral L-Arginine or D-Arginine

| Group      | n | Body wt<br>g | Baseline data |                                        | L-NMMA      |                 |                                        | L-Arginine   |
|------------|---|--------------|---------------|----------------------------------------|-------------|-----------------|----------------------------------------|--------------|
|            |   |              | MAP           | GFR                                    | MAP         | % change in MAP | GFR                                    | MAP          |
|            |   |              | mmHg          | $\mu\text{l/min}/100\text{ g body wt}$ | mmHg        | %               | $\mu\text{l/min}/100\text{ g body wt}$ | mmHg         |
| L-Arginine | 9 | 228 $\pm$ 9  | 117 $\pm$ 2   | 924 $\pm$ 19                           | 169 $\pm$ 2 | +43.9 $\pm$ 1.0 | 1018 $\pm$ 42                          | 116 $\pm$ 2  |
| D-Arginine | 7 | 222 $\pm$ 15 | 158 $\pm$ 3   | 1100 $\pm$ 27                          | 185 $\pm$ 4 | +17.1 $\pm$ 2.2 | 1126 $\pm$ 27                          | 128 $\pm$ 2* |
| P          |   |              | 0.0001        | 0.0001                                 | 0.003       | 0.0001          |                                        | 0.04         |

\*  $P < 0.05$  compared with baseline MAP of the D-arginine group.

More importantly, our data also showed the efficacy of L-arginine in the prevention of hypertension in SS/Jr rats but not in the spontaneously hypertensive strain of rat. Daily intraperitoneal injections of L-arginine or L-citrulline as well as oral L-arginine prevented hypertension in SS/Jr rats (Figs. 1–3). Prevention of hypertension occurred in a dose-response fashion, with doses as low as 25 mg/kg per d achieving some effect. In contrast, parenteral doses of L-arginine, 250 mg/kg per d, did not prevent development of hypertension in spontaneously hypertensive rats (Table V). D-Arginine had no effect on blood pressure regulation in the SS/Jr rats and L-arginine did not lower blood pressure in the SR/Jr rats. In SS/Jr rats, L-arginine also increased urinary cGMP (Fig. 5), another index of NO activity (12). In our hands, after 4 wk on the high-salt diet, SS/Jr rats ( $n = 16$ ) on oral L-arginine were normotensive with blood pressures similar to SR/Jr rats ( $n = 8$ ) on the same diet (SS/Jr rats, 115 $\pm$ 2 mmHg vs. SR/Jr rats, 115 $\pm$ 2 mmHg); 10 of 16 SS/Jr rats on the high-salt diet alone died during the period of observation (unpublished observation). While on the high-salt diet, SR/Jr and Sprague-Dawley rats but not SS/Jr rats demonstrated an augmented blood pressure response to L-NMMA; provision of the substrates for NO restored this augmented blood pressure response to the inhibitor of NO production in SS/Jr rats (Fig. 4). Recognizing there are potential differences in the physiological and biochemical characteristics between SS/Jr and SR/Jr rats, the data, taken together, support the hypothesis that one of the defects producing hypertension in the SS/Jr rats was a reduced NO production which could be corrected with L-arginine.

Table VII. Urinary cGMP Concentrations: Baseline, during Infusion of L-NMMA and after L-Arginine Bolus in Anesthetized SS/Jr Rats after 2 wk on 8% NaCl Chow and Oral L-Arginine or D-Arginine

| Group      | Urine [cGMP]<br>baseline                        | [cGMP] during<br>L-NMMA infusion | [cGMP] after<br>L-arginine bolus |
|------------|-------------------------------------------------|----------------------------------|----------------------------------|
|            | $\mu\text{mol/ml GFR per } 100\text{g body wt}$ |                                  |                                  |
| L-Arginine | 15.0 $\pm$ 1.9                                  | 7.4 $\pm$ 1.9*                   | 26.2 $\pm$ 4.2                   |
| D-Arginine | 9.3 $\pm$ 1.1                                   | 4.7 $\pm$ 1.0*                   | 12.7 $\pm$ 2.9                   |
| P          | 0.03                                            |                                  | 0.03                             |

\*  $P < 0.05$  compared with cGMP values at baseline and after the L-arginine bolus in the same group.

NO synthase has recently been purified by Bredt and Snyder (24). This 150-kD enzyme requires NADPH, calmodulin, and calcium to convert L-arginine to L-citrulline and NO (24). Immunohistochemical studies have localized this NO synthase in the rat to various neurons throughout the brain and to the endothelium of blood vessels. Immunoprecipitation of this protein from solution or addition of benzylnitroarginine, another guanidino nitrogen-substituted arginine molecule, inhibits production of NO (25). Thus, along with several essential cofactors, a single gene product, NO synthase, may be responsible for NO generation by the endothelium.

We conclude that the salt-resistant Dahl/Rapp rats responded to an increase in dietary NaCl by augmenting NO production which served to maintain a constant blood pressure despite a high salt intake. Salt-sensitive Dahl/Rapp rats developed hypertension on the high-salt diet unless provided with exogenous L-arginine, which prevented the hypertensive response. The antihypertensive effect of L-arginine was not non-selective because it did not prevent the increase in blood pressure in the spontaneously hypertensive rat, nor did L-arginine lower established hypertension in these animals. While an abnormality linked to the renin gene locus (26) and renal impairment of salt excretion (27) may also play a role in development of hypertension in these rats perhaps by augmenting salt retention while on a high-salt diet, our data indicate that either L-arginine availability or the activity of NO synthase is abnormal in the salt-sensitive rats. We would suggest that the gene encoding

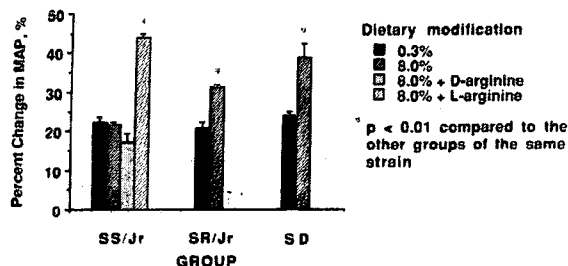
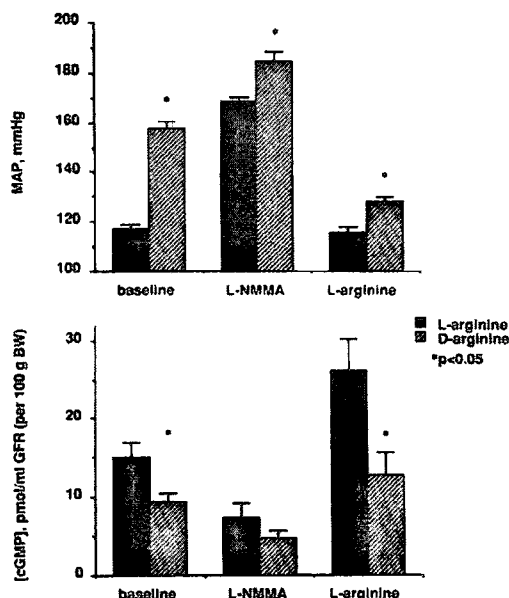


Figure 4. Results of L-NMMA infusion on MAP in SS/Jr, SR/Jr, and Sprague-Dawley (SD) rats. Dietary sodium chloride (0.3 or 8.0%) intake is shown in the legend; two additional groups of SS/Jr rats received oral D-arginine or L-arginine plus the high-salt diet. An increase in dietary sodium chloride increased ( $P < 0.01$ ) the blood pressure response to L-NMMA in the SR/Jr and SD rats, but did not change the response in SS/Jr rats unless they had also received L-arginine.



**Figure 5.** Effects of infusion of L-NMMA followed by L-arginine on blood pressure and urinary cGMP levels in SS/Jr rats on the 8.0% NaCl diet and receiving either L-arginine or D-arginine in their drink for the previous 2 wk. At baseline, blood pressure was lower and urinary cGMP higher in those rats treated with L-arginine. During infusion of L-NMMA, blood pressure increased and urinary cGMP fell acutely in both groups. Mean urinary cGMP during L-NMMA infusion was less ( $P < 0.05$ ) than those values obtained both during baseline and recovery periods. After stopping this infusion and administering a single intravenous bolus of L-arginine, blood pressure rapidly fell and cGMP increased in both groups.

the endothelial form of NO synthase may be a candidate gene involved in the development of hypertension in the Dahl/Rapp salt-sensitive rat. However, as suggested by John P. Rapp (28), cross-breeding studies to show that this gene cosegregates with the hypertensive phenotype will be necessary to confirm this hypothesis. As hypertension persists, the effects of NO on vascular smooth muscle would be impaired further (22, 23), aggravating the hypertension and thus producing a positive feedback loop. The other possibility, that there is a defect in L-arginine handling or production in SS/Jr rats, seems less likely because oral L-arginine and intraperitoneal L-citrulline, an amino acid precursor for L-arginine in endothelial cells in culture (19) and in the kidney, protected SS/Jr rats from hypertension, suggesting the absorptive pathway for L-arginine in the digestive tract and L-arginine production were intact. Recently, Sessa et al. (29) showed that biosynthesis of endothelium-derived relaxing factor by bovine aortic endothelial cells in culture was limited by the availability of intracellular L-arginine. Our study is the first in vivo demonstration that increased substrate availability can overcome a functional defect in NO production. To the extent that these results are applicable to humans, two recent reports confirmed a defect in acetylcholine-stimulated endothelium-dependent relaxation in the brachial arteries of patients with essential hypertension, compared with normotensive controls (30, 31). Endothelium-independent arterial relaxation from infusion of sodium nitroprusside was not altered in these

patients (30, 31). Thus, abnormal NO synthase function may be a major factor producing essential hypertension, especially the salt-sensitive variety, by impairing endothelium-dependent arteriolar relaxation. It remains to be seen if a defect in NO synthase is involved in the development of arteriolar hypertension in defined subsets of human essential hypertension.

## Acknowledgments

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**SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO**  
**DIVISIÓN DE NUEVAS CREACIONES**

2020  
Bogotá, D.C., 01/09/2009

Abogado  
**Emilio Ferrero**  
(Apoderado)

|        |            |           |
|--------|------------|-----------|
| ASUNTO | Radicación | 04 111108 |
|        | Trámite    | 002       |
|        | Evento     | 001       |
|        | Actuación  | 430       |
|        | Oficio     | 10568     |
|        | Folios     | 008       |

|                         |                                     |                               |                                     |
|-------------------------|-------------------------------------|-------------------------------|-------------------------------------|
| Patente de Invención    | <input checked="" type="checkbox"/> | Patente de Modelo de Utilidad | <input type="checkbox"/>            |
| Vía Nacional            | <input type="checkbox"/>            |                               |                                     |
| Vía PCT (fase nacional) | <input checked="" type="checkbox"/> | Cap. I                        | <input type="checkbox"/>            |
|                         |                                     | Cap. II                       | <input checked="" type="checkbox"/> |

Como resultado del examen de fondo, realizado con fundamento en el artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le comunica:

**1. Documentos estudiados**

- 1.1. Descripción: Folios: 44 a 51
- 1.2. Reivindicaciones: Folio: 52  
Folio: 82 Memorial de 19/05/2008
- 1.3. Datos PCT:
- |                             |                 |
|-----------------------------|-----------------|
| Solicitud Internacional:    | PCT/FR03/000507 |
| Capítulo II                 |                 |
| Fecha Sol. Internacional:   | 17/02/2003      |
| Publicación. Internacional: | WO03/087050     |
| Fecha Pub. Internacional:   | 23/10/2003      |
| Fecha Inicio Fase Nacional: | 18/11/2004      |
| Fecha Sol. Nacional:        | 04/11/2004      |
- 1.4. Prioridad: FR02/04847 de 18/04/2002
- 1.5. Respuesta del solicitante: Folios: 77 a 83 Memorial de 19/05/2008

**2. Objeto de la invención**

Título de la solicitud

Nueva sal de perindopril y composiciones farmacéuticas que la contienen

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Reivindicaciones

**Reivindicación 1:** Se reivindica la sal arginina de perindopril y sus hidratos

**Reivindicaciones 2 a 4:** Se reivindican las composiciones que contienen la sal arginina de perindopril en cantidad de 0,2 a 10mg y en combinación con uno o más excipientes en la forma de tableta de liberación inmediata.

Sustento Descriptivo

La invención se relaciona con una nueva sal de perindopril y con composiciones farmacéuticas que las contienen. El perindopril, o ácido (2S)-2-[(1S)-carbetoxibutilamino]-1-oxo-propil-(2S,3aS,7aS)perhidroindol-carboxílico, es un inhibidor de la enzima convertidora de angiotensina I. En general cuando se mencionan las sales de adición con base o ácido farmacéuticamente aceptables por vía de ejemplo se encuentran las sales de sodio o el maleato y durante el desarrollo de este producto se ha encontrado la dificultad de diseñar una sal farmacéuticamente aceptable que no solo tenga buena disponibilidad sino también una estabilidad adecuada para ser propicia para la preparación y almacenamiento de las composiciones farmacéuticas.

Las sales comunes de perindopril como el maleato y la sal de sodio se convierten en aceite una vez entran en contacto con la atmósfera y la sal de terc-butilamina es la única que exhibe mejor estabilidad comparada con las otras formas estudiadas pero los comprimidos obtenidos con dicha sal no alcanzan a presentar una vida media de dos años. Estas restricciones son onerosas y por ello resultaba de gran utilidad ensayar una nueva sal de perindopril con el fin de reducir las restricciones de vidas a la sal de terc-butilamina.

De manera sorprendente se ha encontrado que la sal de arginina de perindopril además de ser nueva, tiene ventajas completamente inesperadas sobre todas las otras sales estudiadas y más especialmente sobre la sal de terc-butilamina de perindopril.

- Una vez catalogada la invención ésta se clasificó en la sección: **A 61 K<sup>31</sup>/404** de la **Clasificación Internacional de Patentes**.

3. Determinación del Estado de la Técnica

Considerando que la fecha de presentación de la primera solicitud de patente de la cual se reivindica prioridad es: 18/04/2002, se procedió a efectuar la búsqueda de documentos relacionados en el estado de la técnica con fecha de publicación anterior a ésta.

| N° | Documento | Título                                           | Fecha de Publicación |
|----|-----------|--------------------------------------------------|----------------------|
| D1 | EP0391462 | "Synergistic compositions containing ketanserin" | 10/10/1990           |

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|           |                  |                                                       |            |
|-----------|------------------|-------------------------------------------------------|------------|
| <b>D2</b> | <b>EP0468929</b> | "Stabilized pharmaceutical compositions"              | 29/01/1992 |
| <b>D3</b> | <b>US4914214</b> | "Process for the industrial synthesis of perindopril" | 03/04/1990 |

Anterioridades D1 a D3 en los folios 84 a 109

#### 4. Análisis de patentabilidad (artículo 14 D. 486)

##### 4.1 Nivel inventivo (artículo18 D. 486)

- Se reivindica la sal arginina de perindopril y sus hidratos, así como sus composiciones farmacéuticas (folio 82).
- El estado del arte previo a la invención presenta los siguientes documentos:

##### Anterioridad D1:

En el folio 86 de la publicación D1 se indica que los Inhibidores de la enzima convertidora de angiotensina pueden formar sales de adición con ácidos o bases, dentro de las que se encuentran: sales de amonio, sales de metales alcalinos como potasio, sodio, magnesio y calcio y sales formadas con bases orgánicas como la benzatina, la N-metil-D-glucamina e hidrabamaina, pero además señala la posibilidad de que formen sales con amino ácidos como arginina y lisina, entre otros.

En el folio 85 se indica que los inhibidores de la enzima convertidora de angiotensina (ECA) son captopril, enalapril, lisinopril, alacepril, indolapril, sirapapril, zofenopril, perindopril y perindoprilat.

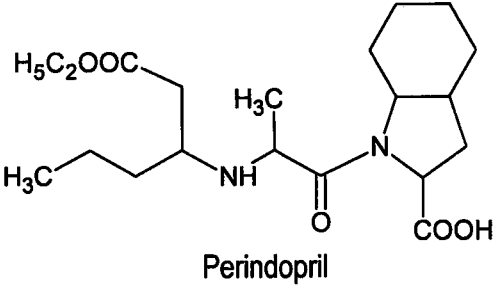
##### Anterioridad D2:

La publicación D2 señala la falta de estabilidad de las composiciones que contienen compuestos inhibidores de la enzima convertidora de angiotensina, por lo que para la preparación de las composiciones pretenden adicionar un componente que sea portador de un ácido de Lewis como clorhidrato de glicina, clorhidrato de ácido glutámico, clorhidrato de betaína, clorhidrato de alanina, clorhidrato de valina, clorhidrato de lisina, clorhidrato de arginina, entre otros.

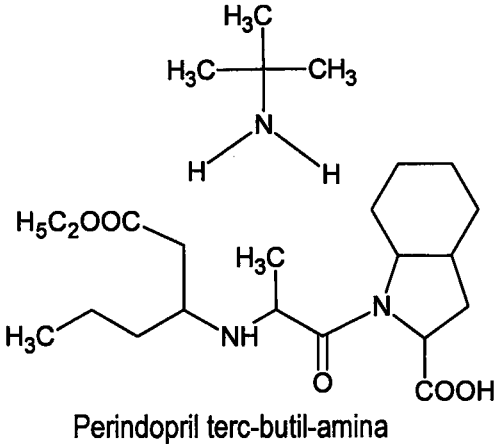
La publicación logra demostrar que por la adición de sustancias que presenten propiedades como ácido de Lewis se logra extender la vida media de las composiciones y por tanto se conserva la estabilidad del producto. Y dentro de los compuestos que señala la anterioridad como inhibidores ECA se encuentra el perindopril (ver folio 96).

##### Anterioridad D3:

La publicación D3 señala un proceso para sintetizar perindopril, en el cual (2S,3aS,7aS)-2-carboxiperindol es condensado con N-[(S)-1carbetoxtibutil]-(S)-alanina después de la desprotección del grupo carboxilo. El producto resultante de la condensación sugiere la desprotección del grupo carboxilo sobre el anillo heterocíclico, para obtener el compuesto de fórmula (I).



La publicación **D3** también hace referencia al proceso para la obtención de la sal de terc-butilamina de perindopril de fórmula (II):



**Evaluación de Diferencias técnicas:**

Al efectuar la comparación elemento por elemento entre el objeto reclamado y las enseñanzas reveladas por el estado del arte más cercano a la invención se hace evidente que el diseño de sales para lograr un producto más estable en el tiempo ya venía siendo objeto de búsqueda por parte de los investigadores en el tema.

**Tabla 1.**

| Materia Reivindicada        | D1                                              | D2                                                                                                                        | D3                                                                                  |
|-----------------------------|-------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|
| Sal arginina de perindopril | Inhibidores ECA pueden formar sales de arginina | Formación de sales en composiciones mediante reacción de inhibidores ECA con ácidos de Lewis como clorhidrato de arginina | Proceso para la obtención de perindopril de fórmula (I) y su sal de terc-butilamina |

Con respecto a cada uno de los documentos **D1** y **D2** la diferencia estriba en que en estos documentos no se precisa que el inhibidor ECA sea perindopril, sino que se refiere de manera genérica al conjunto de moléculas que se agrupan en la categoría terapéutica: inhibidores de la enzima convertidora de angiotensina. Lo que si se indica en estos documentos en la posibilidad de que se formen sales con aminoácidos como es el caso de arginina y en especial el documento **D2** señala la forma como se obtienen in situ las sales de arginina con inhibidores ECA.

Con respecto al documento **D3** la diferencia estriba en que el procedimiento allí consignado sólo hace referencia a la obtención de la sal de terc-butilamina de perindopril, y no se refiere a la formación de la sal de arginina de perindopril.

Si bien es cierto que en la publicación sólo se revelan sales diferentes de perindopril, un conocedor de la materia hubiera podido **D3** diseñar una sal como la reclamada a partir de los conocimientos de conjunto y los saberes revelados en el documento, más si se tiene en cuenta que las diferentes farmacopeas en el mundo establecen

listados de sales aprobadas para administración humana aquellas formadas con aminoácidos esenciales, tal es el caso de Arginina.

**Evaluación del problema técnico objetivo y del efecto técnico:**

De acuerdo con la memoria descriptiva de la solicitud, la invención hace referencia a una nueva sal de adición de perindopril que presenta buena biodisponibilidad y estabilidad.

Debido a que las sales de sodio y maleato modifican su estado de agregación cuando se ponen en contacto con la atmósfera y teniendo en cuenta que la sal de terc-butilamina de perindopril no es estable cuando se somete a condiciones de temperatura y humedad era necesario diseñar una molécula que presentara ciertas ventajas técnicas.

Así, el problema que resuelve la nueva sal de arginina de perindopril se relaciona con la baja estabilidad de las sales convencionales y la necesidad de aumentar la biodisponibilidad del compuesto.

De acuerdo con el señor memorialista el efecto técnico que presentan las sales de arginina tiene que ver con la reducción en la transformación química de la molécula, originada por el menor porcentaje de humedad recibida del medio a medida que se aumenta la temperatura y el aumento en su biodisponibilidad.

Al respecto es preciso señalar que la formación de sales orgánicas como estrategia para mejorar las condiciones de una sustancia con actividad farmacológica, es comúnmente utilizada en farmacia, tan es así que las publicaciones del estado del arte D1 y D2 señalan la posibilidad de que cualquier agente inhibidor de la enzima convertidora de angiotensina ECA pueda formar una sal de arginina.

Por otra parte, es bien sabido por personas versadas en la materia que el objetivo principal de la industria en la etapa de preformulación de un compuesto activo es identificar la forma sólida adecuada para el desempeño de la formulación, la manufactura y su almacenamiento. Por tal razón, la fabricación de sales de adición con ácidos, bases y aminoácidos es una de las estrategias que contemplan los documentos del estado del arte D1 y D2 para mejorar el comportamiento fisicoquímico del compuesto cuando se somete a diferentes condiciones de fabricación y almacenamiento.

Y sin lugar a dudas, para la comunidad científica es claro que la administración de arginina con una molécula inhibidora de la enzima convertidora de angiotensina mejora los resultados respecto de la respuesta terapéutica, toda vez que la arginina es un mediador de la síntesis de factores de relajación del endotelio vascular como es el caso del óxido nítrico, tal y como se evidencia de la lectura de la bibliografía técnica relacionada con el tema<sup>1</sup>.

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<sup>1</sup> Pei Yan Chen. Et al. "L-Arginine abrogates salt-sensitive hypertension in Dahl/Rapp rats". Nephrology research and training center and division of nephrology. Department of Medicine. University of Alabama. Clin Invest. The American Society for Clinical Investigation, Inc. Volume 88, November 1991. 1559-1567.

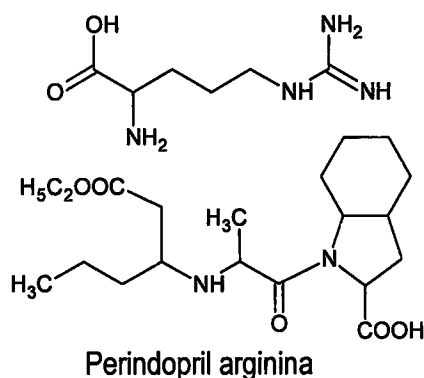
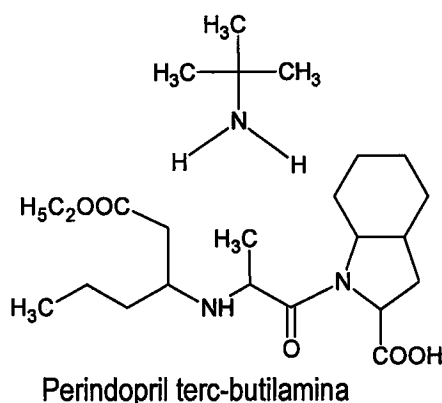
El aminoácido L-arginina presenta actividad como agente hipotensor porque es capaz de normalizar la presión arterial originada por el consumo de sal en la dieta. Este estudio realizado en ratas Dahl/Rapp demuestra que la administración de sal en la dieta aumentó la producción de un importante factor de relajación del endotelio, el óxido nítrico – NO - en los animales, pero esta situación sólo ocurrió en aquellos que fueron tratados concomitantemente con L-arginina, por lo que se logró detener la respuesta hipertensiva (ver folio 114).

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En este orden de ideas, la materia reivindicada en la presente solicitud carece de nivel inventivo ya que tanto las sales como su obtención obedecen a una reproducción, en lo sustancial de los medios utilizados generalmente por la comunidad científica para la producción de sustancias más solubles, estables y fisicoquímicamente superiores y se preveía la posibilidad de que perindopril formara una sal con arginina, tal y como se entiende de la lectura de cualquiera de los documentos **D1** o **D2** analizado en conjunto con **D3**.

Además, se tiene amplio conocimiento de las ventajas terapéuticas que se alcanzan con la administración combinada de un IECA con L-arginina<sup>1</sup>, por lo que para un conocedor de la materia técnica resulta obvio que la formación de una sal de L-arginina con perindopril redunda en una respuesta terapéutica superior y en una biodisponibilidad mejorada, debido a que se conserva la potencia etiquetada.

La formación de una sal tiene como fundamento la interacción del principio activo con una solución que contiene el aminoácido o el electrolito, tal y como lo señalan las enseñanzas del documento **D2** y es tan clara esta situación que para la formación de perindopril terc-butilamina la anterioridad **D3** señala el procedimiento mediante el cual se logra la transformación química, basándose simplemente en la puesta en contacto del principio activo con una solución que contiene la amina con la que se pretende formar la sal.



#### **Resultados del estudio de estabilidad:**

De acuerdo con el señor solicitante, la sal de terc-butilamina se descompone más rápido que la sal de arginina, sin embargo, los ensayos allegados a esta Dependencia con motivo de la presentación de la solicitud no permiten evidenciar cómo se produce la descomposición del producto cuando se utiliza el principio activo en forma de sal de terc-butilamina *versus* la sal de arginina u otras sales convencionales señaladas por el señor memorialista, como es el caso de la sal sódica y el maleato.

Adicionalmente, la sustentación del estudio de estabilidad no permite estimar cómo será el comportamiento del producto a largo plazo en anaquel aún cuando el señor memorialista pretenda prever a partir de un estudio de estabilidad acelerada una vida media de tres años, simplemente mediante extrapolación de los datos obtenidos en el estudio acelerado.

Al respecto es preciso señalar que un estudio de estabilidad serio debe incluir las condiciones especiales que determinaron su realización como el número de muestras analizadas, el número de repeticiones y la clase de estudio realizado (de índole preliminar o estabilidad acelerada y estabilidad en anaquel o de larga duración).

Según la tabla del folio 50 se analizaron 3 muestras, con el fin de determinar 6 meses después de someterlas a condiciones de temperatura y humedad controlada si el principio activo se había descompuesto o había sufrido alguna inestabilidad. Para la determinación de la concentración de principio activo existente en las tabletas de 2,4mg de la sal de arginina de perindopril y de 2,0mg de la sal de terc-butilamina de perindopril, equivalentes a 1,7mg de perindopril, se utilizó el método de cuantificación UV.

De acuerdo con los resultados, en el transcurso de 6 meses contados a partir de su fabricación e inicio del almacenamiento, la sal de arginina presenta un porcentaje de permanencia de perindopril mucho mayor que el exhibido por las muestras que contenían la sal de terc-butilamina.

Tabla 2.

| Condiciones del Estudio 6 meses | Sal de terc-butilamina de perindopril % | Sal de arginina de perindopril % |
|---------------------------------|-----------------------------------------|----------------------------------|
| 25°C 60%HR                      | 101,0                                   | 99,5                             |
| 30°C 60%HR                      | 94,4                                    | 98,1                             |
| 40°C 75%HR                      | 67,2                                    | 98,6                             |

Al respecto es preciso señalar que un estudio de estabilidad acelerada puede extenderse hasta seis meses aunque generalmente se efectúa por un plazo de 90 días, tiempo a través del cual se someten las muestras a condiciones extremas tal y como se presenta en la tabla anexada: 25°C y 60%HR, 30°C y 60%HR, 40°C y 75%HR.

Sin embargo, de la lectura de los datos surgen diversos interrogantes los que fueron señalados por esta Superintendencia en el examen previo, pero el señor solicitante se rehúsa a contestar, como es el caso de: ¿Por qué el porcentaje de principio activo que se preserva en las muestras sometidas a 30°C y 60%HR es mayor que el de las muestras sometidas a 40°C y 75%HR para el caso de la sal de arginina de perindopril?, ¿Acaso las condiciones de humedad del 75% y 40°C de temperatura no son más extremas que las correspondientes a 30°C de temperatura y 60% de humedad relativa?, ¿Cuántas repeticiones del estudio se realizaron para encontrar los datos reportados?, ¿Cuál es el fundamento técnico que le permite extrapolar los datos del estudio acelerado y estimar que la vida media en anaquel es de 3 años?

En consecuencia, y de conformidad con las disposiciones establecidas por el artículo 28 de la norma andina sobre propiedad industrial esta Dependencia se permite señalar que no existe evidencia que permita identificar el verdadero salto cualitativo en la regla técnica, dado que la divulgación realizada del problema técnico y su solución es un mero acercamiento al estudio definitivo que permite llegar a conclusiones como las argumentadas por el solicitante en los folios 44 a 57 de la solicitud.

En el estudio de estabilidad en anaquel muestras representativas del producto deben ser almacenadas a temperatura ambiente y el número de muestras debe permitir la realización de todas las pruebas que deberán ejecutarse durante el estudio, además las muestras son analizadas periódicamente hasta que expire el periodo de validez, es decir 3 años, pero esta situación no se presenta como parte del sustento descriptivo de la solicitud.

Por las razones expuestas anteriormente esta dependencia reitera que el arte previo y los conocimientos de conjunto de una persona versada en el arte técnico afectan la altura inventiva de la materia reclamada, por cuanto D1 o D2 anticipaban la viabilidad de formar sales de arginina para cualquier IECA, dentro de los que se encuentra perindopril y el estado del arte ya enseñaba la forma como se pueden obtener sales

de amina con la molécula antihipertensora según D3. Por consiguiente para una <sup>127</sup> persona versada en la materia técnica obtener una sal de arginina de perindorpil <sup>127</sup> resultaba evidente a partir de las enseñanzas del estado de la técnica, razón por la cual esta Superintendencia señala el incumplimiento de las disposiciones contempladas en el artículo 18 del ordenamiento jurídico andino.

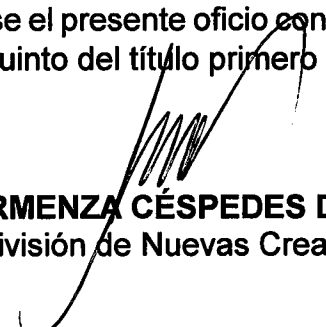
5. Conclusión:

Los requisitos de patentabilidad de la presente solicitud se encuentran afectados así:

| N° | Documento N° | Reivindicaciones Afectadas | Requisito que Afecta |
|----|--------------|----------------------------|----------------------|
| D1 | EP0391462    | 1 a 4                      | Nivel Inventivo      |
| D2 | EP0468929    | 1 a 4                      | Nivel Inventivo      |
| D3 | Artículo 1   | 1 a 4                      | Nivel Inventivo      |

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única N° 10 de 2001.

  
**ALIX CARMENZA CÉSPEDES DE VERGEL**  
Jefe de División de Nuevas Creaciones

El anterior requerimiento fue notificado por fijación en lista, de fecha 11 SET. 2009

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| CONCEPTO TÉCNICO No. 2836 | EXAMINADOR TÉCNICO<br>Código No. 202044 |
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